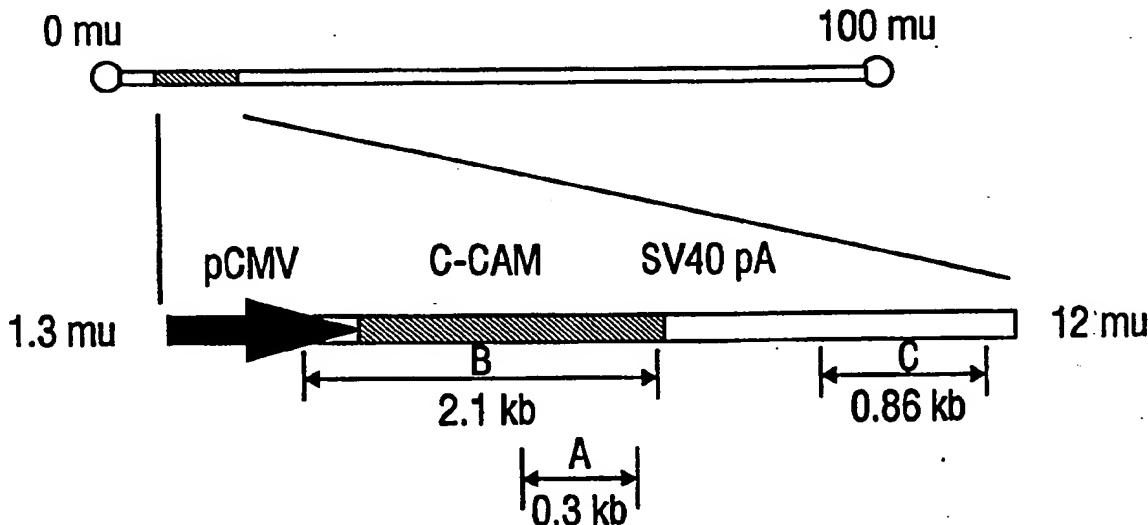




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(54) Title: C-CAM EXPRESSION CONSTRUCTS AND THEIR APPLICATION IN CANCER THERAPY



(57) Abstract

A variety of genetic constructs are disclosed that will find both *in vitro* and *in vivo* use in the area of tumor biology and cancer therapy. In particular, expression constructs are provided that contain a C-CAM encoding region and other regulatory elements necessary for the expression of a C-CAM transcript. One version of the expression construct is a replication-deficient adenoviral vector. Also provided are methods for the transformation of cell lines and the inhibition of cancer cell proliferation.

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DESCRIPTION
C-CAM EXPRESSION CONSTRUCTS
AND THEIR APPLICATION IN CANCER THERAPY

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BACKGROUND OF THE INVENTION

10 1. Field of the Invention

The present invention relates generally to the field of tumor biology. In particular, the invention relates to a nucleic acid encoding a cell adhesion molecule (CAM) and its use in inhibiting tumor growth. In one aspect, the invention relates to expression constructs encoding C-CAM1 and their use in inhibiting prostate and breast cancer.

15 2. Description of the Related Art

Cancers are one of the leading causes of disease, being responsible for 526,000 deaths in the United States each year (Boring *et al.*, 1993). The leading cancer among U.S. men is prostate carcinoma, a disease of epithelial origin. Almost 50% of patients diagnosed with prostate cancer have already developed metastatic lesions at initial clinical presentation, and an additional 20% of patients who initially have local disease will eventually develop metastasis. The standard regimen for metastatic prostate cancer treatment is by androgen ablation. Despite the initial responsiveness to this treatment, tumor cells inevitably relapse to an androgen-independent (AI) state. The mortality associated with this cancer is mostly due to recurrent AI disease for which effective therapeutic regimens are lacking.

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Breast cancer is a major cause of morbidity and mortality in women in the United States, causing about 20% of all cancer deaths in women each year. This disease will develop in about six to seven percent of women in the United States each year, approximately of which half can be cured. Breast cancer is more likely in 5 women who are older, have fibrocystic disease of the breast, have a history of breast cancer in their family, or have had cancer of the endometrium, ovary, or colon. There are several different types of tumors that form in the breast, each with unique characteristics. Most cancers of the breast are detected by the patients themselves, and the average size of the lesion is 2.5 cm. About half of these lesions have already 10 metastasized to the lymph nodes. Early detection by self examination and mammography followed by biopsy to confirm breast cancer is currently the most effective regimen for diagnosis. The complexity of this malignancy thus offers a significant challenge to conventional cancer therapy strategies. Current treatment protocols include combinations of surgical removal of the cancerous tissue, 15 chemotherapy, radiotherapy, and hormone ablation therapy.

With advances in molecular genetics and biology, it has become evident that altered expression of normal genes leads to initiation of cancer cells. The conventional therapy for malignancy, such as chemotherapy and radiation, has focused on mass cell killing without specific targeting, often resulting in damaging 20 side effects. The new direction in cancer therapy is to deliver a normal gene to replace or correct the mutated gene, thereby altering the malignant phenotype of transformed cells. Several expression constructs have been developed in order to deliver a gene into somatic cells with high efficiency.

Cells can be regulated in a positive (stimulatory) or negative (suppressive) 25 manner. Loss of negative regulation of cell growth is often found in malignant cells which exhibit loss of cell proliferation control. Accumulating molecular genetic evidence has revealed that loss of negative regulators or increase in positive regulators in normal cells will produce such cellular growth abnormalities. Most negative regulators (Marx, 1993; Grunicke and Maly, 1993), referred to as tumor suppressors,

have been found to be involved either in direct control of the cell cycle (e.g., Rb, p53, WT-1) or in the signaling pathway leading to cell growth and differentiation (e.g., NF-1). In addition, recent data suggest that genes related to the maintenance of cell architecture and polarity also may function as tumor suppressors (Marx, 1993; Fearon 5 *et al.*, 1990; Trofatter *et al.*, 1993).

Cell adhesion molecules, or CAM's are known to be involved in a complex network of molecular interactions that regulate organ development and cell differentiation (Edelman, 1985). Recent data indicate that aberrant expression of CAM's maybe involved in the tumorigenesis of several neoplasms; for example, 10 decreased expression of E-cadherin, which is predominantly expressed in epithelial cells, is associated with the progression of several kinds of neoplasms (Edelman and Crossin, 1991; Frixen *et al.*, 1991; Bussemakers *et al.*, 1992; Matsura *et al.*, 1992; Umbas *et al.*, 1992). Also, Giancotti and Ruoslahti (1990) demonstrated that increasing expression of $\alpha_5\beta_1$ integrin by gene transfer can reduce tumorigenicity of 15 Chinese hamster ovary cells *in vivo*.

C-CAM is expressed in virtually all epithelial cells (Odin and Obrink, 1987). C-CAM, with an apparent molecular weight of 105 kD, was originally isolated from the plasma membrane of the rat hepatocyte by its reaction with specific antibodies that neutralize cell aggregation (Obrink, 1991). Recent studies indicate that, structurally, 20 C-CAM belongs to the immunoglobulin (Ig) superfamily and its sequence is highly homologous to carcinoembryonic antigen (CEA) (Lin and Guidotti, 1989). Using a baculovirus expression system, Cheung *et al.* (1993a) demonstrated that the first Ig domain of C-CAM is critical for cell adhesive activity.

During liver development, expression of both C-CAM isoforms correlates 25 with differentiation of hepatocytes; the regulation of C-CAM expression is mainly transcriptional (Cheung *et al.*, 1993b; Thompson *et al.*, 1993). Hixson and McEntire (1989) reported an apparent decrease in C-CAM on the cell surface of rat hepatocellular carcinoma lines. In addition, Rosenberg *et al.* (1993) and Neumaier *et al.* (1993) demonstrated that mouse and human biliary glycoprotein, a C-CAM

homologue, is down-regulated in colon tumors. Little is known, however, about the role of C-CAM in cancer progression.

3. Summary of the Invention

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The present invention addresses the need for improved therapy for prostate cancer, breast cancer and other CAM-associated diseases by providing an expression construct containing a nucleic acid encoding a C-CAM. It also is an object of the present invention to provide methods for the use of such compositions and, in 10 particular, use in the treatment of cancer. In another embodiment, the present invention encompasses methods for transforming cells using a C-CAM nucleic acid in an expression construct.

The present invention also encompasses expression constructs that comprises a promoter functional in eukaryotic cells and a nucleic acid encoding a C-CAM, the 15 nucleic acid being under transcriptional control of the promoter.

In a preferred embodiment, the expression constructs further comprise a polyadenylation signal. In another embodiment, the constructs further comprise a selectable marker. In a further embodiment, the expression construct is an adenovirus. In a preferred embodiment, the expression construct is an adenovirus that lacks at 20 least a portion of the E1 region.

In certain embodiments, the nucleic acid is a cDNA. In other embodiments the nucleic acid is a genomic DNA. In an exemplary embodiment the nucleic acid is positioned in a sense orientation with respect to said promoter. In another embodiment the nucleic acid is positioned in an antisense orientation.

25 The present invention also includes pharmaceutical compositions comprising an expression construct with a promoter functional in eukaryotic cells and a nucleic acid encoding a C-CAM, along with a pharmaceutically acceptable buffer, solvent or diluent. In certain embodiments, the expression construct and pharmaceutically acceptable buffer, solvent or diluent are supplied in a kit.

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The invention also provides a method for restoring C-CAM function in a cell that lacks C-CAM function. This method comprises contacting such a cell with an expression construct as described above, wherein the nucleic acid is positioned in a sense orientation. In an exemplary embodiment of the invention, the cell is a 5 transformed cell and the said contacting reverses the transformed phenotype. In a further embodiment, the cell is a prostate tumor cell or a breast tumor cell and, in still a further embodiment, the expression construct is an adenovirus.

The present invention further comprises a method for inhibiting C-CAM function in a cell. This method comprises contacting such a cell with an expression 10 construct as described above, wherein the nucleic acid is positioned in an antisense orientation. In a further embodiment, the expression construct is an adenovirus.

Another embodiment of the invention is a method of treating a mammal with cancer. This method comprises administering to an animal a pharmaceutical composition comprising an expression construct having a promoter functional in 15 eukaryotic cells and a nucleic acid encoding a C-CAM, positioned in a sense orientation, in a pharmaceutically acceptable buffer, solvent or diluent. In a particular embodiment of the invention, the mammal is a human. In another embodiment, administering is via intravenous injection. In a further embodiment, the cancer is prostate, breast or bladder cancer.

20 In further embodiments the present invention encompasses methods for detecting cancer cells in a sample by detecting a C-CAM or a nucleic acid encoding a C-CAM.

4. Brief Description of the Drawings

25 FIG. 1. Linear structure of C-CAM. From the deduced amino acid sequence a linear structure composed of several distinct domains can be predicted. The protein contains two hydrophobic domains, one putative signal sequence in the N-terminal end (indicated by a dashed line) and one transmembrane domain (indicated by a cross-

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hatched rectangle) near the C-terminal end. Four immunoglobulin-like domains can be predicted: the most N-terminal one is a V-like domain, the other three are C-2 domains. The C-2 domains, but not the V-like domain, have cysteine residues that could be involved in intradomain disulfide linkages. A consensus sequence for 5 cAMP-dependent phosphorylation (indicated by a "P" in a circle) is found close to the C-terminal end. The protein contains 16 sites for N-linked glycosylation (indicated by a black dot connected to a line). The location of the glycosylation sites suggests that the major N-terminal portion of C-CAM is exposed on the extracellular side, and that the C-terminal end is located on the cytoplasmic side of the plasma membrane.

10

FIG. 2A. Staining profile of transfected PC-3 cells. PC-3 cells (1×10^4) transfected with C-CAM1 cDNA were stained with Ab669 and FITC-conjugated secondary antibodies and subjected to FACS analysis. The dual-parameter histograms show relative cell size (x-axis), as measured by forward light scatter and log of C-CAM1 expression levels (y-axis), as measured by the intensity of green fluorescence emitted by FITC-stained cells. Positive staining was defined as staining intensity greater than 10 FITC units, 10 FITC units being the background level for cells stained with preimmune serum.

20

FIG. 2B. Staining profile of enriched C-CAM1-positive cell population (cell recovery and cell purity were 93-98% after sorting). PC-3 cells (1×10^4) transfected with C-CAM1 cDNA were stained with Ab669 and FITC-conjugated secondary antibodies and subjected to FACS analysis. The dual-parameter histograms show relative cell size (x-axis), as measured by forward light scatter and log of C-CAM1 expression levels (y-axis), as measured by the intensity of green fluorescence emitted by FITC-stained cells. Positive staining was defined as staining intensity greater than 10 FITC units, 10 FITC units being the background level for cells stained with preimmune serum.

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FIG. 3. Adhesion of C-CAM1 Transfected PC-3 Cells. Single-cell suspensions of PC-3 cells, PC-RSN cells, and C-CAM1 transfected clones were prepared by trypsin digestion of cells from tissue culture plates. One million cells were incubated at room temperature with constant mixing. At the indicated times, the 5 number of single cells was counted with a hemacytometer as described in Materials and Methods, Example II. Legend: PC-3 (open triangles), PC-RSN (closed circles), PC-L1 (open circles), PC-L2 (closed triangles), PC-L6 (closed square), PC-L9 (open square).

10 FIG. 4A. In vitro growth rate of C-CAM1-transfected PC-3 clones as determined by crystal violet staining. Five thousand cells were plated on a 24-well plate in the presence of T medium containing 1% FBS. The medium was changed every 4 days, and the number of cells was determined every 48 hr by the crystal violet assay. Legend: PC-3 (closed circles), PC-RSN (open triangles), PC-L1 (open 15 circles), PC-L2 (closed triangles), PC-L6 (closed square), PC-L9 (open square).

20 FIG. 4B. In vitro growth rate of C-CAM1-transfected PC-3 clones as determined by [³H]thymidine incorporation. Five thousand cells were plated as in FIG. 4A. Fresh medium containing 0.1 mCi of [³H]thymidine was added and, after 2 hr, the cells were harvested and the amount of [³H]thymidine incorporated into the cells was determined. Columns = mean; bars = SD.

25 FIG. 5A. Study 1 - Change in Tumor Volume of C-CAM1-Transfected PC-3 Cells In vivo. Each animal was injected subcutaneously at six sites. The volume of each tumor was recorded weekly when tumors became palpable. Legend: PC-3 (closed circles), PC-RSN (open triangles), PC-L1 (open circles), PC-L2 (closed triangles).

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FIG. 5B. Study 2 - Change in Tumor Volume of C-CAM1-Transfected PC-3 Cells In vivo. Each animal was injected subcutaneously at six sites. The volume of each tumor was recorded weekly when tumors became palpable. Legend: PC-RSN (open triangles), PC-L6 (closed square), PC-L9 (open square).

5

FIG. 6. C-CAM1 recombinant adenovirus construction. A full-length C-CAM1 cDNA (2.1 kB) was first cloned into the *Eco*RI site of the pBSK vector with two possible orientations (sense and antisense). Subsequently, either strand of C-CAM1 insert was directional cloned into the *Hin*DIII (5') and *Not* I (3') sites of 10 pAdE1CMV/pA under the control of a CMV promoter. pJM17 vector contains both viral genome and pBR322 sequences indicated by triangle. Plaques appeared between day 8 and day 10. Each clone of adenovirus was subjected to structural analysis. mu = map unit.

15

FIG. 7. Determination of the genome structure of C-CAM1 adenovirus by PCR. The AdCAM genome of 36 kB is divided into 100 map units (1 mu = 0.36 kB). The C-CAM expression cassette replaced the E1 region (1.3-9.2 mu) of the Ad5 genome. To confirm the structure of AdCAM, three different sets of primers were used. Primer set A is designed to amplify a 300-bp transcript from the C-terminus of 20 C-CAM1 cDNA. Primer set B, including the forward primer from the first intron downstream of human CMV major IE gene promoter and reverse primer from SV40 early polyadenylation signal, is designed to amplify 2.1-kb of the cDNA insert. Primer set C, with two primers localized at 11 and 13.4 mu, is designed to amplify the Ad5 genome-specific sequences (~0.86-kb PCRTM product). mu = map unit.

25

FIG. 8A. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with PBS as a control for 1 day. At the indicated times, the percentage of infected cells was determined using immunofluorescence staining by fluorescent-activated cell scanning analysis. The dual-parameter

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histogram represents relative cell size (x-axis) measured from forward light scatters (FSC) and log of C-CAM1 expression levels (y-axis) measured from the green fluorescence intensity emitted by FITC-stained cells. Positive staining was defined as staining intensity greater than 20 FITC units, which was the background level from control cells as well as the levels from AdCAM101-infected cells stained with antibodies. Fraction of C-CAM-positive cells = 2%.

FIG. 8B. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with AdCAM101 (1,000:1 virions:cell) for 1 day. Fractions of C-CAM-positive cells = 1%.

FIG. 8C. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with AdCAM902 (100:1 virions:cell) for 1 day. Fractions of C-CAM-positive cells = 48%.

FIG. 8D. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with AdCAM902 1,000:1 virions:cell) for 1 day. Fractions of C-CAM-positive cells = 93%.

FIG. 8E. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with PBS as a control for 2 days. Fractions of C-CAM-positive cells = 4%.

FIG. 8F. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with AdCAM101 (1,000:1 virions:cell) for 2 days. Fractions of C-CAM-positive cells = 6%.

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FIG. 8G. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with AdCAM902 (100:1 virions:cell) for 2 days. Fractions of C-CAM-positive cells = 62%.

5 FIG. 8H. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with AdCAM902 1,000:1 virions:cell) for 2 days. Fractions of C-CAM-positive cells = 92%.

10 FIG. 8I. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with PBS as a control for 4 days. Fractions of C-CAM-positive cells = 2%.

15 FIG. 8J. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with AdCAM101 (1,000:1 virions:cell) for 4 days. Fractions of C-CAM-positive cells = 2%.

FIG. 8K. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with AdCAM902 (100:1 virions:cell) for 4 days. Fractions of C-CAM-positive cells = 77%.

20 FIG. 8L. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with AdCAM902 1,000:1 virions:cell) for 4 days. Fractions of C-CAM-positive cells = 95%.

25 FIG. 9. The tumor-suppressing effect of C-CAM1 adenovirus on PC-3 tumors. Fourteen tumors were treated in each group. A single dose of 50 ml of either control (PBS + 10% glycerol) or viral solution (5×10^{11} AdCAM101 or AdCAM902 virions/ml in PBS + 10% glycerol) was injected subcutaneously above the tumor mass 20 days after inoculation with PC-3 cells. Tumor volume was recorded every week

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and tabulated as mean \pm standard deviation (indicated by error bars). Arrow indicates viral injection. Legend: control (closed triangles), AdCAM101 (open circles), AdCAM902 (closed circles).

5 FIG. 10A. Control - Determination of the treatment schedule of C-CAM1 adenovirus for PC-3 tumors. Five tumors (each indicated by a different symbol) were treated in each group. Control: a dose of 50 ml of PBS + 10% glycerol was injected once a week for three consecutive weeks when tumor volume was larger than 40 mm³. Tumor volume was recorded every week. Arrow indicates viral injection.

10

FIG. 10B. AdCAM101 - Determination of the treatment schedule of C-CAM1 adenovirus for PC-3 tumors. Five tumors (each indicated by a different symbol) were treated in each group. 5×10^{11} AdCAM101 virions/ml in PBS + 10% glycerol was injected once a week for three consecutive weeks when tumor volume was larger than 15 40 mm³. Tumor volume was recorded every week. Arrow indicates viral injection.

20 FIG. 10C. AdCAM902 - Determination of the treatment schedule of C-CAM1 adenovirus for PC-3 tumors. Five tumors (each indicated by a different symbol) were treated in each group. A dose of 5×10^{11} AdCAM902 virions/ml in PBS + 10% glycerol was injected once a week for three consecutive weeks when tumor volume was larger than 40 mm³. Tumor volume was recorded every week. Arrow indicates viral injection.

25 FIG. 11. Structure of wild type and mutant C-CAM proteins. The Ig domains are labeled D1 to D4. sig, signal sequence; L, linker domain; TM, transmembrane domain; cyto, cytoplasmic domain.

FIG. 12A. Controls - Effects of C-CAM1 and its mutants on the tumorigenicity of MDA-MB-468 cells *in vivo*. MDA-MB-468 cells were uninfected,

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infected with AdCAM1-AS, or infected with AdCAM1. Twenty-four hours after infection, the cells were injected subcutaneously into nude mice at 10^6 cells per site. Each column represents one tumor site. The tumor sizes were recorded at four weeks.

5 FIG. 12B. Mutants - Effects of C-CAM1 and its mutants on the tumorigenicity of MDA-MB-468 cells *in vivo*. MDA-MB-468 cells were infected with AdCAM1-ΔD1, AdCAM1-H458 or AdCAM1-G454. Twenty-four hours after infection, the cells were injected subcutaneously into nude mice at 10^6 cells per site. Each column represents one tumor site. The tumor sizes were recorded at four weeks.

10

FIG. 13. Aggregation of MDA-MB-468 cells expressing C-CAM1 mutants. MDA-MB-468 cells were infected with AdCAM1 or the mutants indicated, and the cell adhesion assay was performed as described in Example IX. The aggregation of cells is expressed as the decrease in the percentage of single cells.

15

5. Detailed Description of the Preferred Embodiments

The present inventors now have determined that C-CAM can act as a tumor suppressor. In light of this observation, there are at least two uses for C-CAM-related nucleic acids and constructs for their expression. First, in the context of cell culture, it may be possible to generate C-CAM negative cells by targeting endogenous C-CAM with antisense methodology in order to immortalize such cells. This may prove particularly interesting in the generation of cell lines for the study of cancer and, in particular, prostate and breast cancer. Second, the present invention addresses the need for improved therapy for cancer and other C-CAM-associated diseases. In particular, an expression construct capable of expressing a functional C-CAM product can be used to inhibit tumor cell proliferation. The following description provides a more detailed explanation of these and other aspects of the present invention.

C-CAM AND C-CAM-RELATED NUCLEIC ACIDS**A. C-CAM**

The nucleic acid of the present invention may encode an entire C-CAM gene, 5 a functional C-CAM protein domain, or any C-CAM polypeptide, peptide or fragment that is sufficient to affect cell adhesion. The C-CAM nucleic acid may be derived from genomic DNA, *i.e.*, cloned directly from the genome of a particular organisms. In preferred embodiments, however, the nucleic acid encoding C-CAM would comprise of complementary DNA (cDNA). The term "cDNA" used here, is intended 10 to refer to DNA prepared using messenger RNA (mRNA) as template. The advantage of using a cDNA, as opposed to genomic DNA or DNA polymerized from a genomic DNA or non- or partially-processed RNA template, is that the cDNA does not contain any non-coding sequences but, rather, contains only the coding region of the corresponding protein. There may be times when the full or partial genomic sequence 15 is preferred, however, such as where the non-coding regions are required for optimal expression or where non-coding regions such as introns are to be targeted in an antisense strategy. Throughout the application, the term C-CAM is intended to refer to the rat C-CAM or cell-CAM 105, the human C-CAM homologue (biliary glycoprotein 1 (BGP1)), and all C-CAM homologues from other species.

20

(i) C-CAM Protein Structure

C-CAM, like all other known cell adhesion molecules (CAMs), are large intrinsic cell-surface glycoproteins that are mobile in the plane of the membrane (Gall and Edelman 1981; Pollerberg *et al.*, 1986). CAMs mediate cell recognition and 25 adhesion and are of prime importance for the formation and integrity of tissues. The majority of the known CAMs belongs to one of the following families: the immunoglobulin (Ig) superfamily (Williams *et al.*, 1988), the cadherin family (Takeichi, 1988), the integrin superfamily (Hynes, 1987), the LEC-CAM family (Stoolman, 1989) and the H-CAM family (Stoolman, 1989). The cell adhesion

molecule C-CAM belongs to the immunoglobulin superfamily, and more specifically is a member of the carcinoembryonic antigen (CEA) gene family.

The Ig-superfamily is a large family that, in addition to the immunoglobulins, contains many other proteins, the majority being involved in cellular recognition phenomena (Williams *et al.*, 1988). The common building block is the immunoglobulin domain of about 100 amino acid residues that is arranged as a sandwich of two sheets of anti-parallel β -strands. Different members of the superfamily have varying numbers of Ig-like domains. A list of putative CAMs presently known, that belong to the Ig-superfamily, is given in Table 1. One characteristic feature of CAMs in the Ig-superfamily is that they are calcium-independent, that is, they do not need calcium ions for their binding activity. Both homophilic and heterophilic binding occur among the members of this family.

There seem to be several subfamilies of CAMs in the Ig-superfamily. One of these is the carcinoembryonic antigen (CEA) family (Benchimol *et al.*, 1989), which contains a large number of different proteins that can be either transmembrane, GPI-linked, or secreted (Khan *et al.*, 1989). Several of the members of the CEA family cross-react immunologically. It has been reported that both CEA itself, which has seven Ig-domains, and NCA (nonspecific cross-reactive antigen), which has three Ig-domains can function both as homophilic and heterophilic, calcium-independent cell adhesion molecules (Benchimol *et al.*, 1989; Oikawa *et al.*, 1989).

A close examination of the amino acid sequence of rat C-CAM reveals that it is a member of the immunoglobulin superfamily (Aurivillius *et al.*, 1990). Lin and Guidotti noted that the ecto-ATPase/C-CAM is highly homologous to human biliary glycoprotein 1 (BGP1), with 65% of the amino acids being identical (Lin *et al.*, 1989). BGP1 is a member of the CEA-family (Aurivillius *et al.*, 1990; Lin *et al.*, 1989). Thus, it has been concluded that C-CAM belongs to the CEA-subfamily of the immunoglobulin superfamily.

So far, two C-CAM isoforms, C-CAM1 and C-CAM2, have been identified. Both isoforms are composed of a cytoplasmic domain, a 25 amino acid long

transmembrane domain, and an extracellular domain with four Ig-like domains (FIG. 1). Sixteen potential sites for N-glycosylation are found in the extracellular domain, a finding that agrees well with the chemical determination of the carbohydrate content in the mature protein (Obrink 1991). C-CAM1 and C-CAM2 differ in their first Ig domains by 16 amino acids and in the length of their cytoplasmic domains (Celic *et al.*, 1992). C-CAM1, but not C-CAM2, shows adhesion activity when expressed in a baculoviral vector in insect cells (Cheung *et al.*, 1993a). C-CAM binds to itself in a homophilic manner. Further structural and functional analyses of C-CAM1 by Cheung *et al.* (1993a) indicated that the presence of the first Ig domain is essential for adhesion.

Table 1. CAMs in the Ig-superfamily

CAM	Cell Type	Species	References
N-CAM	Neuron, glia muscle	Mammals, birds, amphibians	Williams <i>et al.</i> , 1988 Hansson <i>et al.</i> , 1990
L-1 Ng-CAM	Neuron Ep	Mouse, rat,	Moos <i>et al.</i> , 1988
NILE G4	Gila	chicken	Salzer <i>et al.</i> , 1987
MAG		Man, mouse, rat, chicken, cow	
Po	Schwann	Rat	Lemke <i>et al.</i> , 1988
Contactin	Neuron	Chicken	Ranscht, 1988
F11	Neuron	Chicken	Brümmendorf <i>et al.</i> , 1989
F3	Neuron	Mouse	Gennarini <i>et al.</i> , 1989
Fasciclin II	Neuron	Grasshopper	Harrelson <i>et al.</i> , 1988
Neuroglian	Neuron, glia, many other cell types	Drosophila	Bieber <i>et al.</i> , 1989
Amalgam	Neuron	Drosophila	Seeger <i>et al.</i> , 1988
TAG-1	Neuron	Rat	Furley <i>et al.</i> , 1990
C-CAM	Ep. En. Lc.	Rat, man	Odin <i>et al.</i> , 1988; Tingstrom <i>et al.</i> , 1990; Aurivillius <i>et al.</i> , 1990

Table 1. CAMs in the Ig-superfamily (continued)

CAM	Cell Type	Species	References
CEA	Ep.	Man	Benchimol <i>et al.</i> , 1989
NCA	Ep. Lc.	Man	Oikawa <i>et al.</i> , 1989
DCC	Ep.	Man	Fearoz <i>et al.</i> , 1990
GP42	Fibroblast	Mouse	Altruda <i>et al.</i> , 1989
MUC18	Melanoma	Man	Lehmann <i>et al.</i> , 1989
PECAM1/End	Lc. En. 5M. Trc	Man, cow	Newman <i>et al.</i> , 1990
o-CAM/CD31	En	Man	Elices <i>et al.</i> , 1990
VCAM1/	Lc. many cell	Man, mouse	Staunton <i>et al.</i> , 1988
INCAM-110	types	Man	Staunton <i>et al.</i> , 1989
ICAM1/CD54	En	Man	Streuli <i>et al.</i> , 1988
ICAM-2	Ly. kidney, prostate		
LAR			
CD2	Ly	Man	Williams and Barclay, 1988
LFA-3/CD58	Many cell types	Man	Williams and Barclay, 1988
CD4	Ly	Man, mouse	Williams and Barclay, 1988
CD8	Ly	Man, mouse	Williams and Barclay, 1988
MHC I	All kinds	Man, mouse, etc.	Williams and Barclay, 1988
MHC II	Mo. dendritic	Man, mouse, etc.	Williams and Barclay, 1988
TCR	T-cells	Man, mouse, etc.	Williams and Barclay, 1988
CD28	T-cells	Man	Linsley <i>et al.</i> , 1990
B7/BB-1	B-cells	Man, mouse	Linsley <i>et al.</i> , 1990
B29	B-cells	Mouse	Hermanson <i>et al.</i> , 1988

(ii) C-CAM Location and Function

C-CAM antigens are expressed in a number of organs of mature rats (Odin *et al.*, 1988). The tissue distribution within these organs is, however, limited. Thus, C-CAM is primarily expressed in various epithelia (including hepatocytes of the liver), endothelia of capillaries, small arteries and veins, and in megakaryocytes, platelets,

polymorphonuclear leukocytes and a subset of mononuclear leukocytes (Odin *et al.*, 1988). It has not been found in nervous tissues, muscle tissues or connective tissues.

C-CAM appears rather late in the fetal development of the liver and goes through transient down-regulation during regeneration after partial hepatectomy (Odin *et al.*, 1986). In this context, it is also interesting to note that the expression of C-CAM is significantly altered in hepatocellular carcinomas, so that it is either missing or chemically modified (Hinson *et al.*, 1985; Hinson *et al.*, 1989). In addition, Rosenberg *et al.* (1993) and Neumaier *et al.* (1993) demonstrated that mouse and human biliary glycoprotein, a C-CAM homologue, is down-regulated in colon tumors.

Odin *et al.* (1988) using highly sensitive immunodetection systems were not able to detect C-CAM in the prostate gland nor the epithelium of the urinary bladder. Yet the inventors have not only detected C-CAM in prostate and bladder cells but also identified the role of C-CAM in these cells as a tumor cell suppressor. C-CAM has not been detected in breast cancer cells, yet it is able to act as a tumor suppressor, as disclosed in Example X. The urinary bladder is a musculomembranous sac, situated in the anterior part of the pelvic cavity, that serves as a reservoir for urine which it receives through the ureters and discharges through the urethra. While the prostate is a male gland that surrounds the neck of the bladder and the urethra.

Example V, disclosed herein, demonstrated that C-CAM was associated with basal cells from the normal prostate gland obtained from fetal, juvenile and adult prostate. In contrast, there is a complete disappearance of C-CAM in prostate carcinoma, suggesting that down-regulation of C-CAM is associated with the onset of hyperplastic/neoplastic transformation. Examples I through IV, demonstrated that expression of C-CAM1 suppresses the tumorigenicity of a prostatic cancer cell line, PC-3. Also, using an antisense strategy, endogenous C-CAM expression in a nontumorigenic prostatic epithelial cell line (NbE) was reduced, thereby inducing tumor formation by these cells. In Examples VI and VII, the delivery of C-CAM adenovirus was able to repress the growth of PC-3 induced tumors in mice. Finally,

Example X demonstrated that expression of C-CAM1 suppresses the *in vivo* growth of a breast cancer cell line, MDA-MB-468.

Although a preferred embodiment of the invention is the inhibition of tumor proliferation, transformation and detection of cancer in prostate, breast and bladder
5 cells, any cell or tissue which is affected by C-CAM expression is within the scope of this invention.

(iii) C-CAM Mechanism of Action

Although the mechanisms by which C-CAM affects these processes are not
10 clear, these cell-surface adhesion molecules most likely exert their biological effects through interactions with other cellular molecules, that transduce outside signals into the cells. For example, the integrins, which are Ca^{2+} -dependent CAMs known to interact with a variety of extracellular matrices, have been shown to associate with several intracellular tyrosine kinases (Luna and Hitt, 1992). On the other hand, CAMs
15 have also been shown to participate in the organization of cell architecture by connecting cytoskeleton molecules through an adherens-junction protein complex. Recent molecular genetic analyses (Trofatter *et al.*, 1993) suggest that a potential tumor suppressor gene (NF-2) involved in neurofibromatosis belongs to the family of adherens junction molecules. Also, in the case of E-cadherin, at least three
20 cytoplasmic proteins, α -, β -, and γ -catenin, are associated with the cytoplasmic domain of cadherin (Takeichi, 1991). These associated proteins, which are part of the adherens junction proteins, play important roles in maintaining cellular architecture (Geiger, 1983). Consistent with this observation, recently it was found that α - and β -catenin were also associated with the product of a potential tumor suppressor gene
25 involved in colon carcinogenesis, APC (Rubinfeld *et al.*, 1993; Su *et al.*, 1993). These observations suggest that a cell-adhesion molecule can regulate cell growth through indirect interactions with a tumor suppressor.

The cytoplasmic domain of C-CAM1 contains several potential phosphorylation sites, including one for cAMP-dependent kinase and one for tyrosine

kinase. The putative tyrosine phosphorylation sequence is also within the consensus sequence for the antigen-receptor homology domain (Cambier and Campbell, 1992) that is postulated to be important for signal transduction from membrane-bound IgM molecules in B cells. These structural features suggest that the cytoplasmic domain of C-CAM1 may be critical for signal transduction and that C-CAM1 may interact with other kinases to elicit a negative signal for cell growth. Examples IX and X, disclosed herein, demonstrated that the cytoplasmic domain of C-CAM1 is involved in suppressing growth of the breast tumor cell line MDA-MB-468, and that adhesion does not appear to be required for tumor suppression. It is also possible that the cytoplasmic domain of C-CAM1 interacts with cytoskeletal proteins that modulate cellular morphology.

(iv) C-CAM and Cell Adhesion

While nucleic acid encoding a C-CAM1 (as disclosed herein as SEQ ID NO:1) is the preferred embodiment of the invention, it is believed that nucleic acid encoding C-CAM-related proteins will also function according to the present invention. For example, in certain embodiments, nucleic acids encoding any one of the domains of C-CAM, as seen in FIG. 1 and described above, may function according to the present invention. Thus, one embodiment of the invention may comprise a nucleic acid encoding all or part of the cytoplasmic domain, with or without phosphorylation sites. In another embodiment of the invention, a nucleic acid may encode for all, part or none of the transmembrane portion of C-CAM. In a further embodiment, a nucleic acid may encode for part or all of the extracellular domain, with or without sites for N-glycosylation. In yet a further embodiment, a nucleic acid encoding a domain or peptide from another CAM, such as a member of the Ig superfamily listed in Table 1, may be included in the present invention. It is envisioned that, without undue experimentation, a nucleic acid encoding a CAM may be obtained comprising of several different CAM domains or peptides and still function according to the present invention.

It is also envisioned that a nucleic acid encoding a C-CAM or a related C-CAM may have variations within its sequences that are biological functional equivalents (see below). In order to function according to the present invention the nucleic acid encoding a C-CAM must affect cell adhesion. To test for such an affect, 5 it is a simple matter to assay binding of a protein, encoded by a C-CAM nucleic acid, *in vitro* or by the use of transfection techniques as described in the Examples and disclosed herein.

10 *In vitro* binding assays have been performed on N-CAM and Ng-CAM linked to Covaspheres or other solid substrates, or in artificial lipid vesicles (Hoffman and Edelman, 1983; Sadoul *et al.*, 1983; Grumet and Edelman, 1988) to determine CAM binding kinetics and mechanisms. These methods also may be employed, alone or in conjunction with other methods, to determine the affect of related C-CAMs on adhesion.

15 Preferred methods for testing the affect a nucleic acid encoding a C-CAM has on cell adhesion properties, include the use of transfection studies. Methods for transfecting cells *in vitro* and for assaying cell adhesion and anchorage-independent growth are described in detail in Examples I through III, and IX, while assessment of *in vivo* tumorigenicity is described in Examples III, VI, VII and X.

20 (v) C-CAM Biological Functional Equivalents

As used in this application, the term "nucleic acid encoding a C-CAM" refers to a nucleic acid molecule that has been isolated free of total cellular nucleic acid. In preferred embodiments, the invention concerns a nucleic acid sequence essentially as set forth in SEQ ID NO:1. The term "as set forth in SEQ ID NO:1" means that the 25 nucleic acid sequence substantially corresponds to a portion of SEQ ID NO:1 and has relatively few codons that are not identical, or functionally equivalent, to the codons of SEQ ID NO:1. The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine,

and also refers to codons that encode biologically equivalent amino acids (as in Table 2 below).

Allowing for the degeneracy of the genetic code, sequences that have between about 50% and about 75%; or more preferably, between about 76% and about 99% of 5 nucleotides that are identical to the nucleotides of SEQ ID NO:1 will be sequences that are "as set forth in SEQ ID NO:1". Sequences that are essentially the same as those set forth in SEQ ID NO:1 may also be functionally defined as sequences that are capable of hybridizing to a nucleic acid segment containing the complement of SEQ ID NO:1 under standard conditions. Suitable hybridization conditions will be well 10 known to those of skill in the art and are clearly set forth herein, e.g., see Example I.

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Table 2.

<u>Amino Acids</u>	<u>Codons</u>							
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GU	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

Naturally, the present invention also encompasses DNA segments that are complementary, or essentially complementary, to the sequence set forth in SEQ ID NO:I Nucleic acid sequences that are "complementary" are those that are capable of base-pairing according to the standard Watson-Crick complementary rules. As used herein, the term "complementary sequences" means nucleic acid sequences that are substantially complementary, as may be assessed by the same nucleotide comparison set forth above, or as defined as being capable of hybridizing to the nucleic acid segment of SEQ ID NO:I under relatively stringent conditions such as those described herein.

10 The DNA segments of the present invention include those encoding biologically functional equivalent C-CAM proteins and peptides. Such sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the
15 application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by man may be introduced through the application of site-directed mutagenesis techniques, e.g., to introduce improvements to the adhesiveness of the protein or to test mutants in order to examine signal transducing
20 activity at the molecular level.

If desired, one may also prepare fusion proteins and peptides, e.g., where the C-CAM coding regions are aligned within the same expression unit with other proteins or peptides having desired functions, such as for purification or immunodetection purposes (e.g., proteins that may be purified by affinity
25 chromatography and enzyme label coding regions, respectively).

It will also be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N-or C-terminal amino acids or 5' or 3' sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the 5 maintenance of biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to coding nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region, such as promoters.

As mentioned above, modification and changes may be made in the structure 10 of C-CAM (as exemplified by SEQ ID NO:2) and still obtain a molecule having like or otherwise desirable characteristics. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules, receptors, or signal 15 transduction. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence (or, of course, its underlying DNA coding sequence) and nevertheless obtain a protein with like (agonistic) properties. Equally, the same considerations may be employed to create a protein or polypeptide with 20 countervailing (e.g., antagonistic) properties. It is thus contemplated by the inventors that various changes may be made in the sequence of C-CAM proteins or peptides (or underlying DNA) without appreciable loss of their biological utility or activity.

It is also well understood by the skilled artisan that, inherent in the definition of a biologically functional equivalent protein or peptide, is the concept that there is a limit to the number of changes that may be made within a defined portion of the molecule and still result in a molecule with an acceptable level of equivalent biological activity. Biologically functional equivalent peptides are thus defined herein as those peptides in which certain, not most or all, of the amino acids may be substituted. In particular, where the N-terminal of the C-CAM protein is concerned, it is contemplated that only about 16 or more preferably, about 5 amino acids may be changed within a given peptide. Of course, a plurality of distinct proteins/peptides with different substitutions may easily be made and used in accordance with the invention.

Amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. An analysis of the size, shape and type of the amino acid side-chain substituents reveals that arginine, lysine and histidine are all positively charged residues; that alanine, glycine and serine are all a similar size; and that phenylalanine, tryptophan and tyrosine all have a generally similar shape. Therefore, based upon these considerations, arginine, lysine and histidine; alanine, glycine and serine; and phenylalanine, tryptophan and tyrosine; are defined herein as biologically functional equivalents.

In making changes, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); 25 alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte & Doolittle, 1982, incorporated herein by reference). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still 5 retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ± 2 is preferred, those which are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

It is understood that an amino acid can be substituted for another having a 10 similar hydrophilicity value and still obtain a biologically equivalent protein. As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate ($+3.0 \pm 1$); glutamate ($+3.0 \pm 1$); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); 15 methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

In making changes based upon similar hydrophilicity values, the substitution 20 of amino acids whose hydrophilicity values are within ± 2 is preferred, those which are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

While the preceding discussion has focused on functionally equivalent polypeptides arising from amino acid changes, it will be appreciated that these changes may be effected by alteration of the encoding DNA, taking into consideration also that the genetic code is degenerate and that two or more codons may code for the 25 same amino acid.

In addition to the peptidyl compounds described herein, the inventors also contemplate that other sterically similar compounds may be formulated to mimic the key portions of the peptide structure. Such compounds, which may be termed peptidomimetics, may be used in the same manner as the peptides of the invention and hence are also functional equivalents. The generation of a structural functional equivalent may be achieved by the techniques of modelling and chemical design known to those of skill in the art. It will be understood that all such sterically similar constructs fall within the scope of the present invention.

10 B. Antisense Constructs

In an alternative embodiment, the C-CAM nucleic acid may encode the antisense version of any of the above full-length or fragmentary nucleic acids. The sense or coding constructs will generally be used in methods for inhibiting tumor proliferation where the lack of CAM function is a problem and replacement of CAM function is desired. However, in embodiments where overexpression of CAM is a problem, such as where inhibition or suppression of CAM expression is desired, antisense molecules may be employed.

The term "antisense nucleic acid" is intended to refer to the oligonucleotides complementary to the base sequences of CAM-encoding DNA and RNA. Antisense oligonucleotides, when introduced into a target cell, specifically bind to their target nucleic acid and interfere with transcription, RNA processing, transport and/or translation. Targeting double-stranded (ds) DNA with oligonucleotide leads to triple-helix formation; targeting RNA will lead to double-helix formation.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. Antisense RNA constructs, or DNA encoding such antisense RNAs, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject. Nucleic acid sequences which comprising "complementary nucleotides" are those which are capable of base-

pairing according to the standard Watson-Crick complementarity rules. That is, that the larger purines will base pair with the smaller pyrimidines to form only combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T), in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA.

As used herein, the terms "complementary" or "antisense sequences" mean nucleic acid sequences that are substantially complementary over their entire length and have very few base mismatches. For example, nucleic acid sequences of fifteen bases in length may be termed complementary when they have a complementary 10 nucleotide at thirteen or fourteen positions with only a single mismatch. Naturally, nucleic acid sequences which are "completely complementary" will be nucleic acid sequences which are entirely complementary throughout their entire length and have no base mismatches.

While all or part of the gene sequence may be employed in the context of 15 antisense construction, statistically, any sequence of 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence. Although shorter oligomers are easier to make and increase *in vivo* accessibility, numerous other factors are involved in determining the specificity of hybridization. Both binding affinity and sequence specificity of an oligonucleotide to its 20 complementary target increases with increasing length. It is contemplated that oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more base pairs will be used. One can readily determine whether a given antisense nucleic acid is effective at targeting of the corresponding host cell gene simply by testing the constructs *in vitro* to determine whether the endogenous gene's function is affected or 25 whether the expression of related genes having complementary sequences is affected.

In certain embodiments, one may wish to employ antisense constructs which include other elements, for example, those which include C-5 propyne pyrimidines. Oligonucleotides which contain C-5 propyne analogues of uridine and cytidine have

been shown to bind RNA with high affinity and to be potent antisense inhibitors of gene expression (Wagner *et al.*, 1993).

As an alternative to targeted antisense delivery, targeted ribozymes may be used. The term "ribozyme" is refers to the an RNA-based enzyme capable targeting 5 cleaving particular base sequences in C-CAM DNA and RNA. Ribozymes can either be targeted directly to cells, in the form of RNA oligo-nucleotides incorporating ribozyme sequences, or introduced into the cell as an expression construct encoding the desired ribozymal RNA. Ribozymes may be used and applied in much the same way as described for antisense nucleic acids.

10

EXPRESSION CONSTRUCTS

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being 15 transcribed. The transcript may be translated into a protein, but it need not be. Thus, in certain embodiments, expression includes both transcription of a CAM gene and translation of a CAM mRNA into a CAM gene product. In other embodiments, expression only includes transcription of the nucleic acid encoding a CAM.

20

A. Promoters and Enhancers

In preferred embodiments, the nucleic acid encoding a CAM is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the 25 promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of

several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between elements is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid encoding a CAM is not believed to be important, so long as it is capable of expressing the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In the examples given, the human cytomegalovirus (CMV) immediate early gene promoter (Example VI), the SV40 early promoter and the Rous sarcoma long terminal repeat (Example I), were used to obtain high-level expression of C-CAM. However, the use of other viral or mammalian cellular promoters which are well-

known in the art also is suitable to achieve expression of a CAM, provided that the levels of expression are sufficient for a given purpose.

By employing a promoter with well-known properties, the level and pattern of expression of a CAM following transfection can be optimized. For example, selection 5 of a promoter which is active specifically in prostate cells, such as the prostate-specific antigen (PA) promoter (Watt *et al.*, 1986), will permit tissue-specific expression of a CAM. Further, selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of CAM. For example, with the nucleic acid encoding CAM being expressed from the human PAI-1 10 promoter, expression is inducible by tumor necrosis factor. Tables 1 and 2 list several elements/promoters which may be employed, in the context of the present invention, to regulate the expression of CAM. This list is not intended to be exhaustive of all the possible elements involved in the promotion of CAM expression but, merely, to be exemplary thereof.

15 Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed 20 of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a 25 promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Aside from this operational distinction, enhancers and promoters are very similar entities.

Promoters and enhancers have the same general function of activating transcription in the cell. They are often overlapping and contiguous, often seeming to have a very similar modular organization. Taken together, these considerations suggest that enhancers and promoters are homologous entities and that the 5 transcriptional activator proteins bound to these sequences may interact with the cellular transcriptional machinery in fundamentally the same way.

Below is a list of viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the nucleic acid encoding a CAM in an expression construct (Table 3A and Table 3B). Additionally any 10 promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of a CAM.

Table 3A

ENHANCER
Immunoglobulin Heavy Chain
Immunoglobulin Light Chain
T-Cell Receptor
HLA DQ α and DQ β
β -Interferon
Interleukin-2
Interleukin-2 Receptor
MHC Class II 5
MHC Class II HLA-DR α
β -Actin
Muscle Creatine Kinase
Prealbumin (Transthyretin)
Elastase I
Metallothionein

Table 3A (continued)

ENHANCER
Collagenase
Albumin Gene
α -Fetoprotein
τ -Globin
β -Globin
c-fos
c-HA-ras
Insulin
Neural Cell Adhesion Molecule (NCAM)
α_1 -Antitrypsin
H2B (TH2B) Histone
Mouse or Type I Collagen
Glucose-Regulated Proteins (GRP94 and GRP78)
Rat Growth Hormone
Human Serum Amyloid A (SAA)
Troponin I (TN I)
Platelet-Derived Growth Factor
Duchenne Muscular Dystrophy
SV40
Polyoma
Retroviruses
Papilloma Virus
Hepatitis B Virus
Human Immunodeficiency Virus
Cytomegalovirus
Gibbon Ape Leukemia Virus

Table 3B

Element	Inducer
MT II	Phorbol Ester (TFA) Heavy metals
MMTV (mouse mammary tumor virus)	Glucocorticoids
β -Interferon	poly(rI)X poly(rc)
Adenovirus 5 E2	Ela
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA)
SV40	Phorbol Ester (TFA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
α -2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2kb	Interferon
HSP70	Ela, SV40 Large T Antigen
Proliferin	Phorbol Ester-TPA
Tumor Necrosis Factor	FMA
Thyroid Stimulating Hormone α Gene	Thyroid Hormone

5 B. Selectable Marker

In certain embodiments of the invention, the delivery and expression of a nucleic acid encoding a CAM in a cell may be identified *in vitro* or *in vivo* by including a selectable marker in the expression construct and under the same transcriptional control as the nucleic acid encoding a CAM. The selectable marker would preferably involve a phenotypic change to the transfected cell to easily identify

expression. Usually the inclusion of a drug selection marker aids in the discovery and selection of transformants. Alternatively enzymes such as Herpes Simplex thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be employed. The selectable marker employed is not believed to be important, so long as it is capable of being expressed simultaneously to the nucleic acid encoding a CAM. Further examples of selectable markers are well known to one of skill in the art.

C. Polyadenylation Signal

Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the CAM transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. The inventors have employed the SV40 polyadenylation signal in that it was convenient and known to function well in the target cells employed.

15

D. Viral Expression Vectors

In preferred embodiments of the invention, the expression construct comprises a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells via receptor-mediated endocytosis and to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986). The first viruses used as gene vectors were transforming DNA viruses including the papovaviruses (simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway, 1988; Baichwal and Sugden, 1986). These have a relatively low capacity for foreign DNA sequences and have a restricted host spectrum. Furthermore, their oncogenic potential and cytopathic effects in permissive cells raise safety concerns. Murine retroviruses represent a more attractive alternative to the transforming DNA viruses (Nicolas and

Rubenstein, 1988; Temin, 1986). They can accommodate up to 8 kilobases of foreign genetic material and can be readily introduced in a variety of cell lines and laboratory animals (Nicolas and Rubenstein, 1988; Temin, 1986).

5 (i) Retroviruses

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The 10 integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, gag, pol, and env that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene, termed Ψ , functions as a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are 15 present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a CAM is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and Ψ components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a human cDNA, together with the retroviral LTR and Ψ sequences is introduced into this cell line (by calcium phosphate precipitation for example), the Ψ sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, concentrated, and used for gene transfer. Retroviral vectors are able to

infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the 5 chemical addition of lactose residues to the viral envelope. This modification could permit the specific infection of hepatocytes via sialoglycoprotein receptors.

A different approach to targeting of recombinant retroviruses was designed by Roux *et al.* in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin 10 components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class I antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

There are certain limitations to the use of retrovirus vectors in all aspects of 15 the present invention. For example, retrovirus vectors usually integrate into random sites in the cell genome. This can lead to insertional mutagenesis through the interruption of host genes or through the insertion of viral regulatory sequences that can interfere with the function of flanking genes (Varmus *et al.*, 1981). Another concern with the use of defective retrovirus vectors is the potential appearance of 20 wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact Ψ sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, new packaging cell lines are now available that should greatly decrease the likelihood of recombination (Markowitz *et al.*, 1988; Hersdorffer *et al.*, 1990).

25 One limitation to the use of retrovirus vectors *in vivo* is the inability to produce retroviral vector titers greater than 10^6 infectious U/mL. Titers 10- to 1,000-fold higher are necessary for many *in vivo* applications.

Several properties of the retrovirus have limited its use in prostate cancer treatment (Stratford-Perricaudet and Perricaudet, 1991): (i) Infection by retrovirus

depends on host cell division. In human prostate cancer, very few mitotic cells can be found in tumor lesions (Warner and Heston, 1991). (ii) The integration of retrovirus into the host genome may cause adverse effects on target cells, because malignant cells are high in genetic instability. (iii) Retrovirus infection is often limited by a 5 certain host range. (iv) Retrovirus has been associated with many malignancies in both mammals and vertebrates. (v) The titer of retrovirus, in general, is 100- to 1,000-fold lower than that of adenovirus.

(ii) Adenovirus

10 Knowledge of the genetic organization of adenovirus, a 36 kb, linear and double-stranded DNA virus, allows substitution of a large piece of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the infection of adenoviral DNA into host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal

15 manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in the human.

20 Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range, and

high infectivity. Both ends of the viral genome contain 100-200 base pair (bp) inverted terminal repeats (ITR), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome that 25 contain different transcription units are divided by the onset of viral DNA replication.

The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression, and

host cell shut off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all 5 the mRNAs issued from this promoter possess a 5' tripartite leader (TL) sequence which makes them preferred mRNAs for translation.

In the current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated 10 from this process. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure.

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by AD5 DNA fragments and 15 constitutively expresses E1 proteins (Graham, *et al.*, 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the E3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury, *et al.*, 1987), 20 providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication 25 deficiency of the E1 deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available adenovirus vectors at high multiplicities of infection (Mulligan, 1993).

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such 5 cells include, e.g., Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Other than the requirement that the adenovirus vector be replication defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known 10 serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the method of the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions 15 employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the nucleic acid encoding CAM at the position from which the E1 coding sequences have been removed. However, the position of insertion of 20 the CAM coding region within the adenovirus sequences is not critical to the present invention. The nucleic acid encoding CAM transcription unit may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described previously by Karlsson *et. al.* (1986).

Adenovirus is easy to grow and manipulate and exhibits broad host range *in* 25 *vitro* and *in vivo*. This group of viruses can be obtained in high titers, e.g., 10^9 - 10^{11} plaque-forming unit (PFU)/ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are expressed episomally, and therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination

with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 5 Graham and Prevec, 1992). Recently, animal studies suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1990; Rich *et al.*, 1993). Experiments in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*, 1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et* 10 *al.*, 1993), peripheral intravenous injection (Herz and Gerard, 1993), and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993).

(iii) Other Viral Vectors as Expression Constructs

Other viral vectors may be employed as expression constructs in the present 15 invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988) adeno-associated virus (AAV) (Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984) and herpesviruses may be employed. They offer several attractive features for various 20 mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *In vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et* 25 *al.*, 1990). This suggested that large portions of the genome could be replaced with foreign genetic material. The hepatotropism and persistence (integration) were particularly attractive properties for liver-directed gene transfer. Chang *et al.* recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding

sequences. It was cotransfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.*, 1991).

5

METHODS FOR GENE TRANSFER

In order to effect expression of sense or antisense CAM constructs, the expression construct must be delivered into a cell. This delivery may be 10 accomplished *in vitro*, as in laboratory procedures for transforming cells lines, or *in vivo* or *ex vivo* (see below), as in the treatment of certain disease states. As described above, the preferred mechanism for delivery is via viral infection where the expression construct is encapsidated in an infectious viral particle.

Several non-viral methods for the transfer of expression constructs into 15 cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 20 1979), cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

Once the expression construct has been delivered into the cell the nucleic acid 25 encoding CAM may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding CAM may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the

nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell 5 the nucleic acid remains is dependent on the type of expression construct employed.

In one embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is particularly applicable for transfer *in vitro*, 10 however, it may be applied for *in vivo* use as well. Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of CaPO₄ precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of CaPO₄ precipitated plasmids results in expression of the transfected 15 genes. It is envisioned that DNA encoding a CAM may also be transferred in a similar manner *in vivo* and express CAM.

Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity 20 allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

25 Selected organs including the liver, skin, and muscle tissue of rats and mice have been bombarded *in vivo* (Yang *et al.*, 1990; Zelenin *et al.*, 1991). This may require surgical exposure of the tissue or cells, to eliminate any intervening tissue between the gun and the target organ, *i.e.*, *ex vivo* treatment. Again DNA encoding a

CAM may too be delivered via this method and still be incorporated by the present invention.

In a further embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991).

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful. Using the β -lactamase gene, Wong *et al.* (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa, and hepatoma cells. Nicolau *et al.* (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection.

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear nonhistone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*, then they are applicable for the present invention.

Other expression constructs which can be employed to deliver a nucleic acid encoding a CAM into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific

distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several 5 ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner *et al.*, 1990). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994) and epidermal growth factor (EGF) has also 10 been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.* (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that a 15 nucleic acid encoding a CAM also may be specifically delivered into a cell type such as prostate, epithelial or tumor cells, by any number of receptor-ligand systems with or without liposomes. For example, the human prostate-specific antigen (Watt *et al.*, 1986) may be used as the receptor for mediated delivery of a nucleic acid encoding a C-CAM in prostate cells.

20 In certain embodiments gene transfer may more easily be performed under *ex vivo* conditions. *Ex vivo* gene therapy refers to the isolation of cells from an animal, the delivery of a nucleic acid into the cells, *in vitro*, and then the return of the modified cells back into an animal. This may involve the surgical removal of tissue/organs from an animal or the primary culture of cells and tissues. Anderson *et* 25 *al.* (US 5,399,346), and incorporated herein in its entirety, disclose of *ex vivo* therapeutic methods.

Primary mammalian cell cultures may be prepared in various ways. A preferred method is described, herein (Example I), for a human prostate carcinoma cell line (PC-3). However, many other cell cultures may be established from

tissues/cells in a similar manner, exemplary examples include prostate, breast, bladder, liver and intestines. In order for the cells to be kept viable while *in vitro* and contacting the cells with the expression construct it is necessary to ensure that the cells maintain contact with the correct ratio of oxygen and carbon dioxide and nutrients but are protected from microbial contamination. Cell culture techniques are well documented and are disclosed herein by reference (Freshner, 1992).

During *in vitro* culture conditions the expression construct may then deliver and express a nucleic acid encoding a C-CAM into the cells. Finally the cells may be reintroduced into the original animal, or administered into a distinct animal, in a pharmaceutically acceptable form by any of the means described below. Thus providing an *ex vivo* method of treating a mammal with a pathologic condition.

C-CAM EXPRESSION CONSTRUCTS IN COMBINATION WITH OTHER THERAPIES

Tumor cell resistance to DNA damaging agents represents a major problem in clinical oncology. One goal of current cancer research is to find ways to improve the efficacy of chemo- and radiotherapy by combining it with gene therapy. For example, the herpes simplex-thymidine kinase (HS-tK) gene, when delivered to brain tumors by a retroviral vector system, successfully induced susceptibility to the antiviral agent ganciclovir (Culver, *et al.*, 1992). In the context of the present invention, it is contemplated that C-CAM replacement therapy could be used similarly in conjunction with chemo- or radiotherapeutic intervention.

To kill cells, such as malignant or metastatic cells, using the methods and compositions of the present invention, one would generally contact a "target" cell with a C-CAM expression construct and at least one DNA damaging agent. These compositions would be provided in a combined amount effective to kill or inhibit proliferation of the cell. This process may involve contacting the cells with the C-CAM expression construct and the DNA damaging agent(s) or factor(s) at the same time. This may be achieved by contacting the cell with a single composition or

pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the C-CAM expression construct and the other includes the DNA damaging agent.

- 5 Alternatively, the C-CAM treatment may precede or follow the DNA damaging agent treatment by intervals ranging from minutes to weeks. In embodiments where the DNA damaging factor and C-CAM expression construct are applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the DNA damaging 10 agent and C-CAM expression construct would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would contact the cell with both agents within about 12-24 hours of each other and, more preferably, within about 6-12 hours of each other, with a delay time of only about 12 hours being most preferred. In some situations, it may be desirable to extend the time 15 period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

It also is conceivable that more than one administration of either C-CAM or the DNA damaging agent will be desired. Various combinations may be employed, where C-CAM is "A" and the DNA damaging agent is "B":

20	A/B/A	B/A/B	B/B/A	A/A/B	B/B/B/A	B/B/A/B
	A/A/B/B	A/B/A/B	A/B/B/A	B/B/A/A	B/A/B/A	B/A/A/B
	A/A/A/B	B/A/A/A	A/B/A/A	A/A/B/A	A/B/B/B	B/A/B/B

- 25 To achieve cell killing, both agents are delivered to a cell in a combined amount effective to kill the cell.

DNA damaging agents or factors are defined herein as any chemical compound or treatment method that induces DNA damage when applied to a cell. Such agents and factors include radiation and waves that induce DNA damage such

as, γ -irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, and the like. A variety of chemical compounds, also described as "chemotherapeutic agents", function to induce DNA damage, all of which are intended to be of use in the combined treatment methods disclosed herein. Chemotherapeutic agents 5 contemplated to be of use, include, e.g., adriamycin, 5-fluorouracil (5FU), etoposide (VP-16), camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP) and even hydrogen peroxide. The invention also encompasses the use of a combination of one or more DNA damaging agents, whether radiation-based or actual compounds, such as the use of X-rays with cisplatin or the use of cisplatin with etoposide. In certain 10 embodiments, the use of cisplatin in combination with a C-CAM expression construct is particularly preferred as this compound.

In treating cancer according to the invention, one would contact the tumor cells with a DNA damaging agent in addition to the C-CAM expression construct. This may be achieved by irradiating the localized tumor site with DNA damaging 15 radiation such as X-rays, UV-light, γ -rays or even microwaves. Alternatively, the tumor cells may be contacted with the DNA damaging agent by administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a DNA damaging compound such as, adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, or more preferably, cisplatin. 20 The DNA damaging agent may be prepared and used as a combined therapeutic composition, or kit, by combining it with a C-CAM expression construct, as described above.

Agents that directly cross-link nucleic acids, specifically DNA, are envisaged and are shown herein, to eventuate DNA damage leading to a synergistic 25 antineoplastic combination. Agents such as cisplatin, and other DNA alkylating may be used. Cisplatin has been widely used to treat cancer, with efficacious doses used in clinical applications of 20 mg/m^2 for 5 days every three weeks for a total of three courses. Cisplatin is not absorbed orally and must therefore be delivered via injection intravenously, subcutaneously, intratumorally or intraperitoneally.

Agents that damage DNA also include compounds that interfere with DNA replication, mitosis, and chromosomal segregation. Such chemotherapeutic compounds include adriamycin, also known as doxorubicin, etoposide, verapamil, podophyllotoxin, and the like. Widely used in clinical setting for the treatment of 5 neoplasms, these compounds are administered through bolus injections intravenously at doses ranging from 25-75 mg/m² at 21 day intervals for adriamycin, to 35-50 mg/m² for etoposide intravenously or double the intravenous dose orally.

Agents that disrupt the synthesis and fidelity of nucleic acid precursors, and 10 subunits also lead to DNA damage. As such a number of nucleic acid precursors have been developed. Particularly useful are agents that have undergone extensive testing and are readily available. As such, agents such as 5-fluorouracil (5-FU), are 15 preferentially used by neoplastic tissue, making this agent particularly useful for targeting to neoplastic cells. Although quite toxic, 5-FU, is applicable in a wide range of carriers, including topical, however intravenous administration with doses ranging from 3 to 15 mg/kg/day being commonly used.

Other factors that cause DNA damage and have been used extensively include what are commonly known as γ -rays, X-rays, and/or the directed delivery of 20 radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these factors effect a broad range of damage on the precursors of DNA, the replication 25 and repair of DNA, and the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 weeks), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

The skilled artisan is directed to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 33, in particular pages 624-652. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for

the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

The inventors propose that the regional delivery of C-CAM expression constructs to patients with C-CAM-linked cancers will be a very efficient method for delivering a therapeutically effective gene to counteract the clinical disease. Similarly, the chemo- or radiotherapy may be directed to a particular, affected region of the subjects body. Alternatively, systemic delivery of C-CAM expression construct or the DNA damaging agent may be appropriate in certain circumstances, for example, where extensive metastasis has occurred.

NUCLEIC ACID ENCODING C-CAM OR C-CAM AS MARKERS

In certain embodiments, a nucleic acid encoding a C-CAM or C-CAM may be employed for diagnostic purposes. The absence or reduced/increased level of C-CAM nucleic acid encoding a C-CAM may be indicative of a disease state such as cancer. Thereby using nucleic acid encoding a C-CAM or C-CAM as a marker. There are numerous methods, well known to one of skill in the art, that may be employed in detecting a nucleic acid encoding C-CAM or a C-CAM. Example I, describes two methods for detecting nucleic acids encoding a C-CAM; Southern and Northern analysis. The level of C-CAM message was used as a marker to indicate tumorigenicity.

An alternative approach would be to detect C-CAMs with immunoassays using antibodies that bind to C-CAM. Western Blotting and FACS analysis are also described in Example I. Both techniques were employed to detect C-CAM expression on the cell surface. It will be readily appreciated that detection is not limited to the above techniques, and that there are numerous other methods which may be encompassed by the present invention.

Other, preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISAs) and radioimmunoassays (RIA) known in the art. Immunohistochemical detection using tissue sections is also particularly useful.

- In ELISAs, an anti-C-CAM antibody (such as Ab669, as disclosed herein) is immobilized onto a selected surface exhibiting protein affinity, such as a well in a polystyrene microtiter plate. Then, a test composition containing the cells or cellular material, such as a clinical sample, is added to the wells. After binding and washing to remove non-specifically bound immune complexes, the bound C-CAM may be detected. Detection is generally achieved by the addition of another anti-C-CAM antibody that is linked to a detectable label. This type of ELISA is a simple "sandwich ELISA". Detection may also be achieved by the addition of a second anti-C-CAM antibody, followed by the addition of a third antibody that has binding affinity for the second anti-C-CAM antibody, with the third antibody being linked to a detectable label.
- 15 In another exemplary ELISA, the samples containing the cellular material to be tested for the level of C-CAM, are immobilized onto the well surface and then contacted with the antiC-CAM antibodies. After binding and appropriate washing, the bound immunocomplexes are detected. Where the initial anti-C-CAM antibodies are linked to a detectable label, the immunocomplexes may be detected directly.
- 20 Again, the immunocomplexes may be detected using a second antibody that has binding affinity for the first anti-C-CAM antibody, with the second antibody being linked to a detectable label.

Competition ELISAs are also possible in which test samples compete for binding with known amounts of labeled C-CAM antigens or antibodies. The amount 25 of reactive species in the unknown sample is determined by mixing the sample with the known labelled species before or during incubation with coated wells. The presence of reactive species in the sample acts to reduce the amount of labeled species available for binding to the well and thus reduces the ultimate signal.

Irrespective of the format employed, ELISAs have certain features in common, such as coating, incubating or binding, washing to remove non-specifically bound species, and detecting the bound immunocomplexes. These are described as follows:

In coating a plate with either antigen or antibody, one will generally incubate 5 the wells of the plate with a solution of the antigen or antibody, either overnight or for a specified period of hours. The wells of the plate will then be washed to remove incompletely adsorbed material. Any remaining available surfaces of the wells are then "coated" with a nonspecific protein that is antigenically neutral with regard to the test antisera. These include bovine serum albumin (BSA), casein and solutions of 10 milk powder. The coating allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

In ELISAs, it is probably more customary to use a secondary or tertiary 15 detection means rather than a direct procedure. Thus, after binding of the C-CAM or anti-C-CAM antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the clinical or biological sample to be tested under conditions effective to allow immunocomplex (antigen/antibody) formation. Detection of the 20 immunocomplex then requires a labeled secondary binding ligand or antibody, or a secondary binding ligand or antibody in conjunction with a labeled tertiary antibody or third binding ligand.

"Under conditions effective to allow immunocomplex (antigen/antibody) formation" means that the conditions preferably include diluting the antigens and 25 antibodies with solutions such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween. These added agents also tend to assist in the reduction of nonspecific background.

The suitable conditions also mean that the incubation is at a temperature and for a period of time sufficient to allow effective binding. Incubation steps are

typically from about 1 to 2 to 4 hours, at temperatures preferably on the order of 25° to 27°C, or may be overnight at about 4°C or so.

Following all incubation steps in an ELISA, the contacted surface is washed so as to remove non-complexed material. Washing often includes washing with a 5 solution of PBS/Tween, or borate buffer. Following the formation of specific immunocomplexes between the test sample and the originally bound material, and subsequent washing, the occurrence of even minute amounts of immunocomplexes may be determined.

To provide a detecting means, the second or third antibody will have an 10 associated label to allow detection. Preferably, this will be an enzyme that will generate color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the first or second immunocomplex with a urease, glucose oxidase, alkaline phosphatase or hydrogen peroxidase-conjugated antibody for a period of time and under conditions 15 that favor the development of further immunocomplex formation (e.g., incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween).

After incubation with the labeled antibody, and subsequent to washing to remove unbound material, the amount of label is quantified, e.g., by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethyl- 20 benzthiazoline-6-sulfonic acid [ABTS] and H₂O₂, in the case of peroxidase as the enzyme label. Quantification is then achieved by measuring the degree of color generation, e.g., using a visible spectra spectrophotometer.

PHARMACEUTICAL COMPOSITIONS AND ROUTES OF 25 ADMINISTRATION

Where clinical application of an expression construct comprising a nucleic acid encoding C-CAM is contemplated, it will be necessary to prepare the complex as a pharmaceutical composition appropriate for the intended application. Generally this will entail preparing a pharmaceutical composition that is essentially free of pyrogens,

as well as any other impurities that could be harmful to humans or animals. One also will generally desire to employ appropriate salts and buffers to render the complex stable and allow for complex uptake by target cells.

Aqueous compositions of the present invention comprise an effective amount
5 of the expression construct and nucleic acid, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions can also be referred to as inocula. The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a
10 human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use
15 in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid
20 polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The expression constructs and delivery vehicles of the present invention may include classic pharmaceutical preparations. Administration of therapeutic
25 compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration will be by orthotopic, intradermal subcutaneous, intramuscular, intraperitoneal, or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable

compositions that include physiologically acceptable carriers, buffers or other excipients.

The therapeutic compositions of the present invention are advantageously administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. These preparations also may be emulsified. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. For instance, the composition may contain about 100 mg of human serum albumin per milliliter of phosphate buffered saline. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like may be used. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyloleate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, etc. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components the pharmaceutical composition are adjusted according to well known parameters.

Additional formulations which are suitable for oral administration. Oral formulations include such typical excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. The compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders. When the route is topical, the form may be a cream, ointment, salve or spray.

An effective amount of the therapeutic agent is determined based on the intended goal, for example (i) inhibition of tumor cell proliferation or (ii) elimination of tumor cells. The term "unit dose" refers to physically discrete units suitable for use in a subject, each unit containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses, discussed above, in

association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the subject to be treated, the state of the subject and the protection desired. Precise amounts of the therapeutic composition also depend on the judgment 5 of the practitioner and are peculiar to each individual.

Kits

All the essential materials and reagents required for inhibiting tumor cell proliferation, transforming cells or detecting cancer cells, may be assembled together in a kit. This generally will comprise selected expression constructs. Also included 10 may be various media for replication of the expression constructs and host cells for such replication. Such kits will comprise distinct containers for each individual reagent.

When the components of the kit are provided in one or more liquid solutions, the liquid solution preferably is an aqueous solution, with a sterile aqueous solution 15 being particularly preferred. For *in vivo* use, the expression construct may be formulated into a pharmaceutically acceptable syringeable composition. In this case, the container means may itself be an inhalent, syringe, pipette, eye dropper, or other such like apparatus, from which the formulation may be applied to an infected area of the body, such as the prostate or breast, injected into an animal, or even applied to and 20 mixed with the other components of the kit.

The components of the kit may also be provided in dried or lyophilized forms. When reagents or components are provided as a dried form, reconstitution generally is by the addition of a suitable solvent. It is envisioned that the solvent also may be provided in another container means.

25 The kits of the present invention also will typically include a means for containing the vials in close confinement for commercial sale such as, *e.g.*, injection or blow-molded plastic containers into which the desired vials are retained.

Irrespective of the number or type of containers, the kits of the invention also may comprise, or be packaged with, an instrument for assisting with the injection/administration or placement of the ultimate complex composition within the body of an animal. Such an instrument may be an inhalent, syringe, pipette, forceps, 5 measured spoon, eye dropper or any such medically approved delivery vehicle.

* * * * *

The following examples are included to demonstrate preferred embodiments 10 of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be 15 made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE I: C-CAM1-Transfected PC-3 Cells

A. Materials and Methods

20 1. *Plasmid Construction and Transfection into PC-3 and NbE Cells*

To construct sense and antisense C-CAM expression plasmids, a cDNA insert (1.7 kb) containing the entire coding sequence for C-CAM1 (Lin and Guidotti, 1989; Lin *et al.*, 1991, SEQ ID NO:1) was cloned into the *SaII* site of the vector pRSN (Low 25 *et al.*, 1991), a mammalian expression vector carrying neomycin acetyltransferase gene (*neo*^r), which confers neomycin resistance. The C-CAM1 cDNA was placed under the control of both the Rous sarcoma virus long terminal repeat and the SV40 early promoter. The orientation of C-CAM1 in pRSN was determined by restriction mapping. Digestion of sense clones with BamHI generates four fragments of; 2.7 Kb,

3.4 Kb, 2.1 Kb and 0.7 Kb in length. Digestion of antisense clones generates fragments of; 2.7 Kb, 3.4 Kb, 1.6 Kb and 1.2 Kb in length.

To produce transfected cells, 5 µg of C-CAM1 expression plasmid mixed in 10 µl of Transfectam (Promega, Madison, WI) was added to parental PC-3 or NbE 5 cells grown in T medium (80% Dulbecco's minimum essential medium plus 20% F-12 medium) containing 5% fetal bovine serum (FBS). To select for neomycin-resistant clones, the transfected cells were grown for 1 month in increasing concentrations of G418. The final concentration of G418 was 800 µg/ml and 1 mg/ml for PC-3 cells and NbE cells, respectively.

10

2. Cell Sorting by Flow Cytometry

Membrane fluorescence staining was performed on a single-cell suspension with an anti-C-CAM polyclonal antibody (Ab669; Culic *et al.*, 1992) and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Cheung *et al.*, 1993). FACS 15 was performed with a dual-laser Vantage flow cytometer (Becton Dickinson, Mountain View, CA) delivering 50 mW at 488 nm with an Enterprise air-cooled laser. The flow cytometer was sterilized by a 30-min run with 70% ethanol and equilibration with sterile Isoton II (Coulter, Hialeah, FL) for 1 hr. Log green fluorescence was measured with a bandpass filter (530 nm/30 nm), and triggering mode was executed 20 with forward light scatter set at a sufficiently high threshold to eliminate cell debris and dead cells (<5%). Drop delay was performed using the Autosort procedure (Becton Dickinson, Mountain View, CA). Analysis was performed using LYSYS II software (Becton Dickinson, Mountain View, CA).

25

3. Southern, Northern, and Western Blot Analyses

For Southern analysis, high-molecular-weight DNA was purified by the procedure of Davis *et al.* (1986). Twenty micrograms of high molecular weight DNA was digested with *Hind*III overnight at 37°C and then subjected to Southern blot analysis as described previously (Hsieh *et al.*, 1993) with a C-CAM cDNA probe

(Celic *et al.*, 1992) or a neomycin acetyltransferase (neo^r) cDNA probe (Southern and Berg, 1982).

For Northern analysis, total cellular RNA was extracted from cells by using RNAzol B (Biotecx Laboratories Inc., Houston, TX) and a single-step purification 5 protocol described by Chomczynski and Sacchi (1987). Twenty micrograms of RNA was subjected to Northern blot analysis by electrophoresis on a 0.9% agarose gel containing 2 M formaldehyde as described previously (Hsieh and Lin, 1994), and the blot hybridized with a random primer-labeled C-CAM cDNA probe (Celic *et al.*, 1992), neo^r cDNA probe or 28S RNA probe.

10 To isolate total cellular protein for Western analysis, the cells were trypsinized into a single-cell suspension and counted with a hemacytometer. Aliquots of the cell suspension from each clone were boiled in sodium dodecyl sulfate sample buffer, and Western blot analysis performed as described previously (Cheung *et al.*, 1993) with anti-C-CAM antibody Ab669.

15

B. Results

1. C-CAM1-Transfected PC-3 Cells

To examine the role of C-CAM in prostate cancer progression, the C-CAM1 cDNA in the mammalian expression vector pRSN was transfected into a human 20 prostate carcinoma cell line designated PC-3. This cell line is highly tumorigenic and found to be negative for C-CAM by both Northern and Western analyses. After transfection, the cells were grown in medium containing G418 to select for neomycin-resistant clones.

Immunofluorescence staining of the transfected PC-3 cells with anti-C-CAM 25 antibody Ab669 was performed after 1 month of G418 selection. Although the immunofluorescence staining showed that C-CAM1 was expressed on the cell surface, only a small portion of cells (about 10%) reacted with the polyclonal antibody against C-CAM (FIG. 2A). This result suggested that only a small population of the transfected cells expressed C-CAM1. To increase the proportion of C-CAM1-positive

cells, the C-CAM1-positive cell population was enriched to 92% by fluorescence-activated cell sorting (FACS) (FIG. 2B). Two clones, PC-L1 and PC-L2, were isolated from the enriched population and characterized. Two additional clones, PC-L6 and PC-L9, were later isolated from this enriched population by using the same
5 approach.

Southern blot analysis of genomic DNA isolated from various transfectants, digested with *Hind*III and hybridized with the neomycin acetyltransferase (*neo'*) cDNA probe, revealed several different DNA integration patterns. As expected, the parental PC-3 cells were negative for *neo'*. PC-3 cells transfected with the PC-RSN
10 vector alone (designated PC-RSN cells) showed a complex pattern of *neo'* integration. Two patterns of integration were seen among the four C-CAM1-positive clones, PC-L1 and PC-L2 having one type of integration and PC-L6 and PC-L9 another. These data indicate that these four C-CAM1-positive clones were derived from at least two different cells.

15 No C-CAM message was detected by Northern blot analysis in PC-3 or PC-RSN cells, suggesting that C-CAM was either not expressed or expressed in very low levels in these cells. In PC-L1 and PC-L2 cells, a single 3.0 kB C-CAM1 mRNA was detected. Two C-CAM transcripts were found in PC-L6 and PC-L9 cells, the 3.0 kB transcript and a slightly larger one. This larger transcript may represent a fusion of
20 the *C-CAM* and *neo'* genes, because this band hybridized to both the C-CAM and Neo' probes. As predicted, the *neo'* mRNA was expressed in all transfected clones but not in the parental PC-3 cells. Hybridization with 28S rRNA, which served as an internal control, showed that similar amounts of RNA were loaded for each sample.

Western blot analysis with anti-C-CAM antibody Ab669 revealed similar
25 profiles of C-CAM protein expression in all four C-CAM1 transflectants, while PC-3 and PC-RSN cells were negative for C-CAM expression. The C-CAM1 protein expressed from PC-3 transflectants was heterogeneous; the 105 kDa form was predominant. The heterogeneity may be due to different degrees of glycosylation since there are 16 potential N-linked glycosylation sites present in the extracellular

domain of C-CAM1. More C-CAM1 protein was detected in PC-L1 and PC-L2 cells than in PC-L6 and PC-L9 cells, suggesting that C-CAM1 protein levels may be regulated differently in these clones.

5 **EXAMPLE II - Cell Adhesion and Growth Characteristics of Transfected PC-3 Cells**

A. Materials and Methods

1. *Cell Adhesion Assay*

Cells were resuspended at a concentration of 1×10^6 /ml. Cell suspensions (1
10 ml) in 1.5-ml Eppendorf tubes were mixed gently at room temperature to allow
formation of aggregates. Samples were taken over a 5-hr period, and the number of
single cells was determined with a hemacytometer. The aggregation of cells was
monitored as a decrease in the percentage of single cells.

15 2. *In Vitro Growth Assay*

The *in vitro* growth rate of C-CAM1-transfected PC-3 clones was measured
with three different assays. In one method, cells were grown in T-medium containing
5% FBS, trypsinized and counted with a hemacytometer 24, 48, and 96 hr after
plating. In a second method, 5,000 cells from each clone were grown on a 24-well
20 plate with T-medium containing 1% FBS. The medium was changed every 4 days.
The number of cells was determined every 48 hr using the crystal violet assay as
described in Gleave *et al.* (1991) and incorporated herein by reference. In a third
method, cells were plated as above and every 24 hours, fresh medium containing 0.1
mCi of [³H]-thymidine was added. [³H]-thymidine incorporation was determined
25 after a 2-hr incubation as described in Chang and Chung (1989) and incorporated
herein by reference.

B. Results

To test the intercellular adhesion of C-CAM1 expressed on the transfected PC-3 cells, the ability of single cells to form aggregates in suspension was measured. The time course of aggregate formation for PC-3, PC-RSN, PC-L1, PC-L2, PC-L6, and 5 PC-L9 cells, as measured by the decrease in the number of single cells, is shown in FIG. 3. PC-3 and PC-RSN cells, which did not express the C-CAM1 protein, did not aggregate. In contrast, all of the C-CAM1 transfectants formed aggregates. These results indicate that the C-CAM1 expressed in PC-3 cells was able to elicit adhesion. Consistent with their higher level of C-CAM1 protein expression, PC-L1 and PC-L2 10 cells aggregated slightly more than PC-L6 and PC-L9 cells did.

The effect of C-CAM1 on cell growth also was analyzed. The four C-CAM1 transfected clones had growth rates (measured by counting the cells with a hemacytometer) of about 50% that of control cells. Similar results were obtained by measuring the increase in the number of cells by crystal violet staining (FIG. 4A) and 15 by measuring the rate of DNA synthesis by [³H]thymidine incorporation (FIG. 4B). Crystal violet staining showed that PC-RSN cells had a growth rate similar to that of parental PC-3 cells. In contrast, the growth rates of the C-CAM1-transfected cells were about 25% that of the control cells 6 days after plating (FIG. 4A). By day 8, PC-L6 and PC-L9 cells were growing faster than PC-L1 and PC-L2 cells, perhaps because 20 of the lower C-CAM1 levels in PC-L6 and PC-L9 cells. The rate of DNA synthesis, measured by [³H]thymidine incorporation, was noticeably lower than control levels in all of the C-CAM1-transfected cells (FIG. 4B). This finding suggests that the growth inhibition of C-CAM1-transfected cells may result from an alteration in the cell cycle.

EXAMPLE III- Suppression of Tumorigenicity of PC-3 Cells by C-CAM1 Expression

A. Materials and Methods

1. *Measurement of Anchorage-Independent Growth*

5 The soft-agar assay described by Giancotti and Ruoslahti (1990), and incorporated herein by reference, was used to measure the anchorage-independent growth of each transfected clone. Cells were plated onto 6-well plates, and colonies were scored in triplicate by two different investigators, 21 days after plating, by counting aggregates (>10 cells) in several randomly chosen areas.

10

2. *Assessment of In Vivo Tumorigenicity*

To determine the tumorigenicity of each transfected clone, 1×10^6 cells/site were injected at six sites in the flanks of 8- to 10-week-old male nude mice subcutaneously. Once a tumor became palpable, it was measured weekly and its size 15 calculated using the formula for the volume of a hemiellipsoid, the geometric figure most nearly approximating the shape of the tumor: volume = length x width x height x 0.5236 (Rockwell *et al.*, 1972).

B. Results

20 Normal cells must attach to a substratum to grow, whereas tumorigenic cells do not. As a result of this property, they can form colonies on soft-agar plates (Shin *et al.*, 1975). To determine the effect of C-CAM1 expression on soft-agar colony formation by PC-3 cells, the inventors examined the anchorage-independent growth of the C-CAM1-transfected clones. These four clones formed significantly fewer 25 colonies in soft-agar in three independent studies (Table 4A). In addition, the colonies of PC-L1, PC-L2, PC-L6, and PC-L9 cells were smaller than those of PC-3 and PC-RSN cells. These observations indicate that expression of C-CAM1 may affect the tumorigenicity of PC-3 cells.

**Table 4A. Anchorage-Independent Growth
of C-CAM1-Transfected PC-3 Cells^a**

Clone	Anchorage-independent growth (% of control)		
	Exp 1	Exp 2	Exp 3
PC-3	ND	100 ^b	100 ^b
PC-RSN	100 ^b	100±2	89±3
PC-L1	52±2	45±1	ND
PC-L2	36±4	45±1	30±1
PC-L6	ND	ND	50±3
PC-L9	ND	ND	60±2

5 ^aAnchorage-independent growth was measured by triplicate counts of the colonies that had formed by day 21 after plating. The results are expressed as percentages of control (PC-RSN or PC-3) ± standard error. ND, not determined.

^bControl, defined as 100%.

10 Next, the ability of these cells to produce tumors in nude mice *in vivo* was measured. PC-3 cells previously have been shown to be highly tumorigenic when injected into nude mice (Kaighn *et al.*, 1979). To test the transfected cells, cells from each clone were injected into the flanks of male athymic nude mice subcutaneously and the incidence of tumor formation and the volumes of the tumors monitored. As 15 shown in Table 4, 60 days after injection, the tumor incidence was 67-94% for PC-3 cells and PC-RSN cells. In contrast, a much lower tumor incidence was observed in mice injected with C-CAM1-transfected cells. In addition, parental PC-3- and PC-RSN-induced tumors generally arose within 3 to 4 weeks of injection, whereas injecting the same number of C-CAM1-transfected cells into nude mice produced 20 fewer or no tumors within the first 30 days (Table 4B). In addition, the tumors produced from transfected cells were much smaller (FIG. 5A and FIG. 5B). These

data indicate that expression of C-CAM1 in PC-3 cells suppressed or delayed tumor growth *in vivo*.

**Table 4B. Tumor Incidence Induced by
C-CAM1-Transfected PC-3 Cells**

Clone	<u>Tumor incidence^a(%)</u>			
	<u>Test 1</u>	<u>Test 2</u>	Day 30	Day 60
PC-3	12/36(33%)	24/36(67%)	ND	ND
PC-RSN	29/36(80%)	34/36(94%)	12/18(67%)	17/18(94%)
PC-L1	2/36(5%)	12/36(33%)	ND	ND
PC-L2	0/36(0%)	2/36(5%)	ND	ND
PC-L6	ND	ND	0/18(0%)	0/18(0%)
PC-L9	ND	ND	0/18(0%)	4/18(22%)

^aTumors larger than 10 mm³ were considered to be positive. ND, not determined.

Taken together, these results demonstrated that expression of C-CAM1 markedly suppressed the tumorigenicity, as well as the tumor growth, of PC-3 cells.

10

EXAMPLE IV - Increased Tumorigenicity of NbE Cells by Transfection of Antisense C-CAM1 Plasmid

A. Materials and Methods

1. *In Vitro Characterization of Mac Clones*

15

Plasmid construction and transfection of NbE cells were as described in Example I. Individual Mac clones derived from NbE cells transfected with antisense C-CAM1 cDNA were characterized for cDNA integration and protein expression. For Southern blot analysis, 20 µg of high-molecular-weight DNA was isolated from each cell line, digested with *Hin*DIII at 37°C overnight, and then subjected to 20 Southern blot analysis. The blot was hybridized with a neo' probe and a C-CAM

probe as described in Example I. Total protein (5 µg) from each cell line was subjected to Western blot analysis with the anti-C-CAM antibody, Ab669. The relative levels of C-CAM protein was determined by densitometrical scanning.

After growing in 1 mg/ml G418 for 1 month, transfected cells (antisense-1, 1 x 5 10^6 cells/site) were injected into nude mice subcutaneously.

2. *In Vitro Cell Cloning of NbE Sublines (Mac)*

Tumors were excised from animals and placed in serum-free T-medium containing antibiotics. After removing excess skin and blood clots, tissues were 10 rinsed with serum-free T-medium, 3 times. Tissues were cut into 3 mm³ pieces and placed in a 6-well plate coated with FBS; then, a small amount of 10% FBS T-medium was added to cover each tumor for overnight incubation at 37°C. The medium was changed every 4 days and G418 (200 µg/ml) was added when epithelia started appearing on the dish. The concentration of G418 was gradually increased to a 15 final concentration of 1 mg/ml within 1 month. The cells were then further cloned by the ring-cloning technique as described in Freshney (1987) and incorporated herein by reference.

B. Results

20 To further examine the tumor-suppressor activity of C-CAM1 in prostate cancer development, the inventors tested whether down-regulation of C-CAM in a non-tumorigenic cell line could lead to tumor formation in nude mice. The NbE cell line, a prostatic epithelial cell line derived from the ventral prostate of a Noble rat (Chung *et al.*, 1989), was chosen because it expresses detectable levels of C-CAM and 25 does not cause tumor formation when injected into nude mice or into the syngeneic host (Chung *et al.*, 1989). The antisense C-CAM1 plasmid, constructed by inserting the full-length C-CAM1 cDNA into pRSN in the anti-sense orientation, was used to reduce C-CAM1 expression.

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In the first study, NbE cells were transfected with an antisense C-CAM1 vector. Transfected cells were selected by increasing concentrations of G418. After growing in 1 mg/ml G418 for 1 month, transfected cells (antisense-1, 1 x 10⁶ cells/site) were injected into nude mice subcutaneously. Sixty days later, tumors 5 appeared at all the antisense-1 injection sites (Table 5A), but no tumors appeared where parental NbE cells had been injected. The antisense-induced NbE tumors were excised from the mouse and cells were cloned from these tumors. Since antisense-transfected NbE cells are resistant to G418 treatment and the cells derived from the nude mouse are not, the antisense-transfected NbE cells can be specifically selected 10 from total tumor tissues by G418 selection. After G418 selection for one month, six sublines (named Mac-1 to Mac-6) were isolated from six individual tumors. The tumorigenicities of these six cell lines were tested together with several control NbE cell lines, as shown in Table 5B.

To confirm the previous finding, the same antisense vector was transfected 15 into NbE cells to generate a second transfected NbE cells (antisense-2). Other control NbE cell lines, including those transfected with sense and vector controls, also were generated and selected under the conditions used in the first study. When these cell lines were injected into nude mice, the antisense vector induced tumors at an incidence of 50% appeared 40 days after injection (Table 5B), with an average tumor 20 volume similar to that of the previous study (Table 5A), whereas no tumors developed in mice injected with sense and vector controls (Table 5B).

**Table 5A. Tumor Incidence and Tumor Growth Induced by
Antisense C-CAM1-Transfected NbE Cells**

NbE clones	Tumor incidence (%)	Tumor volume (mm ³)
	60 days	60 days
parental	0/6 (0%)	0
antisense-1	6/6 (100%)	49±9

**Table 5B. Tumor Incidence and Tumor Growth Induced by
Antisense C-CAM1-Transfected NbE Cells**

NbE clones	Tumor incidence ^a (%)		Tumor volume (mm ³)	
	14 days	40 days	14 days	40 days
parental	0/18 (0%)	0/18 (0%)	0	0
plasmid control	0/18 (0%)	0/18 (0%)	0	0
sense	0/18 (0%)	0/18 (0%)	0	0
antisense-2	0/18 (0%)	9/18 (50%)	0	58±8
Mac-1	11/12(92%)	12/12(100%)	34±4	144±25
Mac-2	11/12(92%)	12/12(100%)	45±7	117±14
Mac-3	12/12(100%)	12/12(100%)	37±4	98±16
Mac-4	12/12(100%)	12/12(100%)	50±5	285±37
Mac-5	12/12(100%)	12/12(100%)	47±3	91±13
Mac-6	12/12(100%)	12/12(100%)	44±6	117±14

5 ^aTumors larger than 10 mm³ were considered to be positive.

- Histopathologic examination of hematoxylin- and eosin-stained tumor tissues from these antisense-2 tumors revealed that they were anaplastic and poorly differentiated. In the same *in vivo* tumorigenicity study, all six anti-sense sublines, 10 Mac-1 to Mac-6, showed aggressive tumor formation with almost 100% tumor incidence, and palpable tumors 14 days after inoculation. These results indicate that transfection of antisense C-CAM1 into NbE cells changed the cells from non-tumorigenic to highly tumorigenic.
- Southern analyses were used to determine the clonality of antisense NbE-derived sublines, Mac-1 to Mac-6. All Mac clones hybridized with both the neo' and the C-CAM probes; however, two patterns of integration were observed. Mac-1, Mac-2, Mac-5, and Mac-6 had the same DNA integration pattern, while Mac-3 and

Mac-4 had a different pattern. These results indicate that these six sublines originated from two different clones.

Western immunoblotting indicated that the levels of C-CAM were significantly (10- to 100-fold) lower in all six sublines than in the parental cells.

- 5 Results from the densitometrical scanning, were as follows: parental (100%), plasmid control ($85\pm7\%$), Mac-1 ($9\pm1\%$), Mac-2 ($3\pm1\%$), Mac-3 ($4\pm1\%$), Mac-4 ($1\pm0\%$), Mac-5 ($1\pm0\%$), Mac-6 ($6\pm1\%$). Thus, the increased tumorigenicity of the antisense C-CAM-transfected cells correlated with their reduced C-CAM levels.

10 **EXAMPLE V - Expression of C-CAM During Human Prostate Development**

A. Materials and Methods

1. *Immunohistochemistry*

Tissue samples from radical prostatectomy (25 patients) and transurethral resection of the prostate (1 patient) were obtained from the Department of Pathology at the M.D. Anderson Cancer Center. The tissue samples were immediately placed in tissue blocks with O.C.T. compound (Miles, Elkhart, IN) and frozen at -20°C. Both prostate carcinomas and normal prostatic tissues were included in this study. Serial 4 μ m-thick sections were cut from each sample with a cryostat. One section was stained with hematoxylin and eosin for pathological evaluation and grading; adjacent sections were used for immunohistochemical staining. The prostate adenocarcinomas were graded according to the Gleason grading system. Prostatic intraepithelial neoplasia (PIN) was classified as low grade (PIN1) and high grade (PIN2 and PIN3) as agreed at the 1989 Workshop on Prostatic Dysplasia (Drago *et al.*, 1989). In addition, prostate samples representative of prostate gland development, such as fetal prostates (30- and 36-week-old) and a prostate from a 13-year-old boy were included in the study. These samples were provided as frozen sections by Sherwood *et al.* (1991).

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For immunoperoxidase staining, tissue was placed on silicone coated slides, which were then rinsed in phosphate-buffered saline (PBS). The tissue was fixed in a solution of 95% methanol and 5% acetic acid at -20°C for 15 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol at room 5 temperature for 15 min. After the tissue was washed with PBS three times for 5 min. each, Superblock (Scytek Laboratories, Logan, UT) was applied for 20 min. at room temperature. Slides were then incubated with the polyclonal antibody specific against C-CAM (Ab 669, 1:2000 dilution in PBS containing 1% bovine serum albumin) overnight at 4°C. The negative controls were incubated with preimmune serum. A 10 biotin-avidin amplification system using a StrAviGen™ Super Sensitive kit (Biogenex Laboratories, San Ramon, CA) with diaminobenzidine (DAB) as the chromogen was applied. A light green dye (Sigma Chemical Co., St. Louis, MO) was used for background counterstaining.

Immunochemical staining was evaluated by three observers. The pattern 15 of staining was classified as positive with a continuous staining (staining in a continuous pattern surrounding any given gland), positive with a discontinuous staining (staining in a broken or discontinuous pattern surrounding any given gland), or negative staining (no staining at all).

**Table 6: C-CAM Staining in the Normal Adult Prostate,
BPH, PIN (A) and Primary Prostate Carcinomas (B)**

(A)

Histology	Zonal Origin	Cases	Staining Pattern	
			Continuous ^a	Discontinuous ^b
Normal	Peripheral	20	20 (100%)	0
Normal	Central	2	2 (100%)	0
Normal	Transition	7	7 (100%)	0
Atrophy	Peripheral/ Transition	17	17 (100%)	0
BPH	Transition	5	2 (40%)	3 (60%) ^c
PIN ^d	Peripheral	12	7 (58%)	5 (42%) ^e

5 (B)

Gleason Score	Zonal Origin	Cases	Positive	Negative
5-7	Transition	3	0	3 (100%)
	Peripheral	16	0	16 (100%)
8-10	Peripheral	1	0	1 (100%)
	Not determined ^f	2	0	2 (100%)
Total		22		22 (100%)

^aContinuous: Uniform staining throughout the entire basal cell layer of each gland.^bDiscontinuous: Non-uniform staining of the basal cell layer surrounding each gland.^cIn all 3 of these cases the inventors observed a mixed staining pattern: some BPH glands had discontinuous staining, whereas other BPH glands had negative staining.10 ^dHigh grade PIN (PIN2 and PIN3).^eIn 4 of 5 of these cases the inventors observed a mixed staining pattern: some PIN glands had discontinuous staining, whereas other PIN glands had continuous staining.

In the fifth case of PIN there was solely discontinuous staining.

^fZone not able to be determined.

B. Results

Arising from the urogenital sinus and the vesicourethral components of the cloaca. The bud stage (20-30 weeks gestation) is characterized by solid epithelial buds at the ends of ducts without a recognizable lumen (Xia *et al.*, 1990). Using cell type-specific cytokeratin antibodies for either basal or luminal cells, Sherwood *et al.* clearly showed that only basal cells were present in these epithelial buds (1991). By 36 weeks of gestation, some buds are still visible; however, the majority have developed into acinotubular structures with identifiable lumens (Xia *et al.*, 1990).

To understand the expression pattern of C-CAM during prostate development, the inventors studied the same 30-week- and 36-week-old fetal prostate specimens by immunohistochemical staining using a polyclonal antibody specific against C-CAM, Ab 669. Recent data indicated that Ab 669 raises against rat C-CAM molecule can cross react with the human counterpart BGP-1 (Gao and Knowles, 1993) but appears not to cross-react with CEA and other immunoglobulin-like CAM's (Gao and Knowles, 1993). In the 30-week-old fetal prostate, C-CAM expression could be detected as typical plasma membrane staining associated with multiple basal cell layers in the epithelial buds. In contrast, the surrounding stromal compartment stained negatively.

In addition to epithelial buds in the 36-week-old fetal prostate, there were acino-tubular structures with identifiable lumens. C-CAM expression localized predominantly in the basal cell layers of these maturing glands. Luminal epithelium showed weak if any staining. Furthermore, tissue obtained from a 13-year-old prostate exhibited a continuous positive staining of C-CAM associated with the basal cell layer, and again the luminal epithelium showed weak if any staining. In contrast, the antibody controls showed a negative C-CAM staining. In the normal adult prostate gland, C-CAM expression showed the same positive staining pattern in the basal cell layer as was observed in the fetal prostates. Taken together, these data

indicate that C-CAM expression in the normal human prostate gland is associated with basal cells throughout the early fetal stage into adulthood.

In contrast, the rat ventral prostate shows a different staining pattern; steady-state levels of C-CAM are detected in the luminal epithelium from intact animals.

- 5 However, elevated levels of C-CAM are detected in the basal cell layer only after androgen deprivation (Hsieh and Lin, 1994). This difference in C-CAM staining between the two species may be due to differences in the proliferative potential of luminal epithelium. For example, in the intact rat prostate, proliferative activity is found in the luminal epithelium (English *et al.*, 1987). In the human prostate,
- 10 however, the majority of proliferative activity is confined to the basal cell population (Bonkhoff *et al.*, 1994).

Consistent with the results from fetal prostate development, the inventors observed a continuous plasma membrane staining of C-CAM predominantly associated with basal cells in the normal glandular epithelium from the peripheral, central and transition zones of the prostate. As summarized in Table 3A, the inventors observed the same continuous staining pattern of C-CAM in the basal cell layer in all specimens examined (29 cases) regardless of zonal origin. On the other hand, luminal cells showed weak if any staining. In addition, atrophic glands (17 cases) with a distinct basal cell layer exhibited a continuous C-CAM staining pattern in the basal

15 cell layer in any given gland (Table 3A).

2. *Decreased Expression of C-CAM in BPH and PIN*

Benign prostatic hyperplasia (BPH), often found in the transition zone/periurethral gland region of the human prostate (McNeal, 1978), is considered a

25 hyperplastic growth of epithelium and/or mesenchyme. In this study, an overall decrease in C-CAM staining was observed in the epithelial compartment from BPH glands compared with adjacent normal glands in the transition zone. The overall C-CAM staining pattern of basal epithelium from BPH glands can be divided into two categories. First, as shown in Table 3A, in 40% of specimens examined, the basal

epithelium from BPH glands showed a continuous staining pattern of C-CAM with a similar staining to normal glands, but the majority (60%) of specimens showed either a discontinuous staining pattern in the basal layer or no staining at all. However, in the adjacent normal (nonhyperplastic) glands from the same tissue section, the basal 5 cell layers exhibited a continuous C-CAM staining. Therefore, decreased C-CAM expression may be associated with hyperplasia of the prostatic epithelium.

Prostatic intraepithelial neoplasia (PIN) is considered a precursor of prostate carcinoma (McNeal and Bostwick, 1986; Troncoso *et al.*, 1989). Therefore, it is important to determine C-CAM expression in PIN. The inventors' finding on PIN are 10 limited to high grade (PIN2 and PIN3) lesions that could be reproducibly identified on the frozen sections. Because low grade PIN (PIN1) could not be unequivocally distinguished from epithelial hyperplasia or other minor epithelial atypias on frozen sections, it was not included in the inventors' evaluation.

As summarized in Table 3A, the overall C-CAM staining pattern associated 15 with basal cells is altered in high grade PIN glands. For example, although some PIN glands still exhibited a continuous C-CAM staining pattern in the basal cell layer, adjacent PIN glands had a discontinuous pattern of C-CAM staining along the basal cell layer. Very often, this discontinuous pattern of C-CAM staining associated with PIN was seen adjacent to areas of carcinoma, which were completely negative for C- 20 CAM staining (FIG. 4A and FIG. 4B). Notably, the inventors also observed a mixed pattern of both continuous and discontinuous staining associated with the basal layer within a single PIN gland.

These observations suggested that the altered and decreased C-CAM expression may be associated with the continuous progression from normal prostatic 25 epithelium to premalignant transformation and finally to carcinoma (Bostwick and Brawer, 1987). Moreover, these results indicated that altered C-CAM expression may be an early event associated with prostate cancer development.

3. *Absent Expression of C-CAM in Primary Prostate Carcinomas*

Prostate cancer is a multifocal disease with a wide range of histologic patterns and biologic potential. The heterogeneous staining pattern of C-CAM in premalignant PIN glands prompted the examination of C-CAM expression in prostate cancers of different histologic grade and zonal origin. In all, 22 prostate carcinoma specimens examined, a complete absence of C-CAM staining was observed regardless of tumor grade and zonal origin (FIG. 4 and Table 3B). The carcinomas studied were moderately and poorly differentiated tumors (Gleason score 5-10). Although the inventors did not examine well-differentiated carcinomas (Gleason score 2-4), areas of histologic grade 2 present focally among the moderately differentiated tumors also were negative.

In contrast, normal glands from the same sections still retained a continuous staining pattern of C-CAM, indicating that the absence of staining in carcinoma was not due to staining artifact (FIG. 4C and FIG. 4D). All pre-immune serum controls were negative. Thus, the expression of C-CAM inversely correlated with the appearance of carcinoma. The complete loss of C-CAM even in the foci within moderately-differentiated tumors suggests that the loss of C-CAM occurs early in malignant transformation.

Consistent with the absence of C-CAM expression in human prostate cancer specimens, C-CAM also was not detected in several established human prostate cancer cell lines, including PC-3 (Example I), and DU-145 and LNCaP cells.

EXAMPLE VI - Construction and Analysis of the C-CAM1 Recombinant

Adenovirus

25 A. Materials and Methods

1. *Construction and large-scale production of the C-CAM1 recombinant adenovirus*

To construct a recombinant adenovirus containing the C-CAM1 gene, as shown in FIG. 6, C-CAM1 cDNA (Lin and Guitti, 1989; Cheung *et al.*, 1993a; SEQ

ID NO:1) was cloned into the *Eco*RI site of the multiple cloning sites of the pBSK vector (Stratagene, La Jolla, CA). Either a sense or antisense strand of C-CAM1 cDNA was directionally cloned into the *Hin*DIII and *Not*I sites of a shuttle vector (pAdE1CMV/pA). So that its expression was under the control of a cytomegalovirus (CMV) promoter, one of the very strong promoters (Boshart *et al.*, 1985). The recombinant adenovirus was generated from cotransfection of the shuttle vector (30 µg) with the C-CAM1 cDNA insert and a pJM17 vector (20 µg) carrying both adenoviral genome (Ad5) and pBR322 sequences.

The two vectors were cotransfected into 293 cells using DOTAP reagent (Boehringer-Mannheim, Indianapolis, IN) according to the manufacturer's protocol. The pBR322 sequences in pJM17 make the entire viral DNA too large to be packed into protein coat, which prevents the appearance of wild type virus (Graham and Prevec, 1991). In this study, two replication-deficient recombinant viruses, one containing the sense orientation of C-CAM1 cDNA (AdCAM902) and the other containing the antisense orientation of C-CAM1 cDNA (AdCAM101), were generated from homologous recombination in a packaging cell line (293 cells). Each clone of viruses was isolated from a single plaque after reinfecting 293 cells (Graham and Prevec, 1991). Subsequently, a large amount of viral stocks were produced from 293 cells and purified by Inland Laboratories, Inc. (Austin, TX). The average titers of viral stocks were estimated to be 1×10^{11} virions/ml as determined by A_{260} assay; or 1×10^{11} plaque forming units (pfu/ml) for AdCAM101 and 1×10^{10} pfu/ml for AdCAM902 as determined by a plaque assay.

2. Analysis of the structure of the recombinant adenovirus by PCR

Adenoviral DNA was prepared as described previously (Zhang *et al.*, 1993). PCRTM (Polymerase chain reaction) was performed in a 50-ml reaction mixture containing 4 mM MgCl₂, 50 mM KCl, 0.1% Triton[®] X-100, 200 mM of each deoxyribonucleotide triphosphate, 10 mM Tris-HCl (pH 9.0), 2 mM of the different primer sets (primer set A [^{5'}GATCCAACACAAGGAAATT^{3'}],

5'TCACTTCTTTGACTAC^{3'}]; primer set B [5'GGCCCACCCCTGGCTTC3',
5'TTGTAAACCATTATAAGCTGC3']; or primer set C
[5'TCGTTCTCAGCAGCTGTG3', 5'CATCTGAACCAAAGCGTGG3']), and
1.0 unit of Taq DNA polymerase (Promega, Madison, WI). The reactions were
5 carried out for 30 cycles, and each cycle was performed at 94°C for 0.5 min, at 56°C
for 0.5 min, and 72°C for 1 min.

3. *Determination of viral infectivity of PC-3 cells by Fluorescent-activated cell scanning analysis*

10 PC-3 cells (5×10^5 cells) were plated on a 65-mm plate for 24 h, then different concentrations of viruses were added and incubated at 37°C for 24 h. Cells were trypsinized into a single-cell suspension and incubated with either pre-immune serum or anti-C-CAM polyclonal antibody (Ab669) and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies as described by Cheung *et al.*, 1993a. Percentage of
15 positive cells was determined by a dual-laser Vantage flow cytometer (Becton Dickinson, Mountain View, CA) as described in Example I.

4. *Determination of C-CAM1 expression by C-CAM1 recombinant adenovirus using Northern and Western analyses*

20 To determine the levels of C-CAM1 expression in viral-infected cells, the inventors performed both Northern and Western assays as described in Example I. For the dose-dependent studies, PC-3 cells (5×10^5 cells) were infected with either AdCAM101 or AdCAM902 at various ratios of virions:cells (0:1, 1:1, 5:1, and 20:1) for 24 h. Cells were harvested for the determination of C-CAM1 mRNA and protein
25 levels. For the time course studies, the same amount of PC-3 cells was infected with either AdCAM101 or AdCAM902 at a 5:1 virion:cell ratio and medium was changed every 3 days. For the Northern blot analysis, cells were harvested for the determination of C-CAM1 mRNA levels at day 4, 7, 10 and 14. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. For the Western

blot analysis, cells were harvested for the determination of C-CAM1 protein levels at day 6, 14 and 20.

B. Results

5 To ensure the completion of homologous recombination and the presence of C-CAM in these recombinant adenoviruses, the inventors performed PCRTM using different sets of primers as described in Materials and Methods. As shown in FIG. 7, using a C-CAM-specific primer set (*i.e.*, primer set A) from the C-terminal portion (from amino acid 413 to amino acid 519) of C-CAM (Lin and Guidotti, 1989), the
10 inventors were able to amplify a 0.3 kB transcript from both AdCAM101 and AdCAM902 DNA, indicating that both adenoviruses contained the C-CAM sequences.

Also, using primer set B for the flanking sequences from the human CMV promoter and SV40 polyadenylation site, a 2.1 kB full-length C-CAM cDNA
15 transcript was detected in both viruses. These data indicated that the C-CAM insert was located between CMV promoter and SV40 polyadenylation. Moreover, using an adenovirus-specific primer (*i.e.*, primer set C), an expected 0.86 kB PCRTM transcript appeared from both viruses, indicating that both viruses were generated from homologous recombination. Taken together, these data indicate that these two viruses
20 contain the expected gene structure.

To characterize the infectivity of adenovirus to prostatic cancer cells, PC-3 cells (5×10^5 cells) were plated in a 65-mm dish overnight prior to infection, then various ratios of viral particles per cell were added to cells and incubated at 37°C for 1, 2, and 4 day. The efficiency of viral infection was determined by fluorescent-
25 activated cell scanning based on positive membrane staining of C-CAM1. As shown in FIG. 8, the background fluorescence was set at 20 FITC units based on the scanning of PC-3 cells infected with buffer control (FIG. 8A, FIG. 8E, FIG. 8I). PC-3 cells infected with AdCAM101 (FIG. 8B, FIG. 8F, FIG. 8J) gave the same fluorescence profile as for the buffer control indicating that cells infected with

AdCAM101 did not express C-CAM protein. On the other hand, C-CAM was expressed by PC-3 cells infected with AdCAM902 (FIG. 8C, FIG. 8D, FIG. 8G, FIG. 8H, FIG. 8K, FIG. 8L).

When the inventors determined the percentage of positive cells after 5 AdCAM902 infection for one day, the inventors found that 48% of cells expressed C-CAM1 when the cells were infected at a 100:1 virion:cell ratio (FIG. 8C), and 93% of cells expressed C-CAM when infected at a 1000:1 ratio (FIG. 8D), which is comparable to other report using β -gal adenovirus (Li *et al.*, 1993). These data indicate that C-CAM1 expression in AdCAM902-infected PC-3 cells is dose-dependent. On the other hand, the inventors observed that maximal infection (~93%) 10 plateaued within 24 h at the saturated viral concentration (FIG. 8D, FIG. 8H, FIG. 8L). However, under the subsaturated viral concentration, the infectivity rate increased from 48% at day 1 to 77% at day 4 in a time-dependent manner (FIG. 8C, FIG. 8G, FIG. 8K), suggesting that this recombinant adenovirus can maintain its 15 infectivity at least 4 days.

When PC-3 cells were infected with sense recombinant adenovirus (AdCAM902), both Northern and Western blot analyses showed that C-CAM1 expression could be detected 24 h after infection and that the level of C-CAM1 expression depended on the number of viruses used for infection. Detection of C-CAM expression was easily observed at ratios of 5:1 and 20:1, virions:cell. A much 20 weaker signal was detected from PC-3 cells infected with AdCAM101, using a C-CAM1 cDNA probe, suggesting the presence of the antisense C-CAM1 message. Since antisense mRNA, in general, has a short half-life, this weak signal is expected. AdCAM101 showed no C-CAM1 protein expression, however. These results indicate 25 that the sense recombinant adenovirus (AdCAM902) is capable of expressing C-CAM1. From the time course study, a continuous elevation of C-CAM1 mRNA was detected in viral-infected PC-3 cells 14 days after infection. Similarly, the C-CAM1 proteins were still detectable 20 days after infection. Since the viral genome was not detected 10 days after infection, these data suggest that the prolonged C-CAM1

expression in sense recombinant virus-infected cells may be due to the high stability of C-CAM1 protein.

EXAMPLE VII - Tumor Suppressing Effect of C-CAM1 Recombinant

5 Adenovirus

A. Material and Methods

1. *Measurement of PC-3 tumor growth by in vivo administration of recombinant adenovirus*

To determine the efficacy of C-CAM1 adenovirus on the growth of PC-3 tumors, the inventors injected 1×10^6 cells/site at four sites in the flanks of 8- to 10-week-old male nude mice subcutaneously. Once the tumor became palpable, 50 μ l of either recombinant adenovirus or buffer control (Phosphate-buffered saline + 10% glycerol) were injected near the tumor area (*i.e.*, subcutaneously above the tumors). The change in tumor volume was measured weekly and calculated using the formula described previously in Example III.

B. Results

Previous results, in Example II, indicated that increased C-CAM1 expression in PC-3 cells reduces the *in vitro* growth of this cell line. Based on the *in vitro* time course of C-CAM expression in virus-infected cells (Example VI), the inventors tested the therapeutic efficacy of C-CAM1 on preexisting PC-3 tumors by injecting (50 μ l) of either sense or antisense C-CAM1-adenovirus or buffer control into the area near the tumor mass. The growth of tumors was then monitored weekly. As shown in FIG. 9, the growth of PC-3-induced tumors was suppressed by C-CAM1 adenovirus treatment for at least a 3-week period. At week 6, the inventors observed that tumors regained the growth activity. The second dose (50 μ l) of virus delivered at week 7 was able to continue tumor suppression for at least a 2-week period (FIG. 9). In the same study, during the first few weeks of viral injection, the inventors observed a small degree of tumor inhibition by antisense (AdCAM101) adenovirus treatment.

suggesting that the virus itself may cause some toxic effect on cells since the titer of AdCAM101 was 10-fold higher than that of AdCAM902. However, from the AdCAM101-treated group, the growth of PC-3 induced tumors eventually resumed and the size of tumors were similar to that of the control (FIG. 9).

- 5 Since C-CAM1 adenovirus can suppress the growth of small tumors, the inventors further examined the efficacy of AdCAM902 treatment on larger tumors, ranging from 40 mm³ to 75 mm³, which represent more heterogenous tumors. Based on results in previous examples, the inventors estimated that the effective viral dosage needed to increase at least 3-fold in order to inhibit the growth of larger tumors.
- 10 However, it is difficult to deliver such a large volume (*e.g.*, 150 µl) to tumors at once; therefore, the viruses were equally divided into three dosages and delivered every week for 3 consecutive weeks. In this study, the inventors observed that AdCAM902 (FIG. 10C), but not control (FIG. 10A) or AdCAM101 (FIG. 10B), was still able to suppress the growth of PC-3 tumors for at least 5 weeks starting from the first viral
- 15 injection (time 0). In some cases, however, AdCAM902 failed to suppress the tumor growth if the tumor size reached more than 100 mm³.

EXAMPLE VIII - Construction and Analysis of Recombinant Adenoviruses

- This example shows the construction and analysis of recombinant adenoviruses containing a C-CAM1 cDNA and the effects of C-CAM1 expression on the *in vivo* tumorigenicity of MDA-MB-468 breast cancer cells.

A. Materials and Methods

1. Construction of recombinant adenoviruses

- To study the effects of C-CAM1 expression on the *in vivo* tumorigenicity of the breast cancer cell line MDA-MB-468, a series of adenovirus recombinants were made, as shown in FIG. 11, containing wild-type, anti-sense, or mutant C-CAM cDNA. Recombinant adenoviruses containing the full-length wild-type C-CAM1 cDNA in the sense (AdCAM1) and antisense (AdCAM1-AS) orientations were constructed as described previously (Kleinerman *et al.*, 1995). To construct the

AdCAM1-ΔD1 virus, a 1.5-kb *Hind*III-*Not*I fragment containing the C-CAM1 cDNA lacking the first Ig domain was isolated from pCR1000-ΔD1 (Cheung *et al.*, 1993a) and inserted into an adenoviral shuttle vector, XCMV, to generate the plasmid XCMV-CAM1-ΔD1. The recombinant adenovirus AdCAM1-ΔD1 was generated by 5 co-transfection of the plasmids XCMV-CAM1-ΔD1 and pJM17, a vector that contains the adenovirus genome, into the human embryonal kidney cell line 293 as described previously (Kleinerman *et al.*, 1995).

To construct the AdCAM-H458 virus, the CAM1-H458 cDNA fragment with flanking *Hind*III-*Not*I sites was generated by PCRTM amplification with oligo 42 10 (GTCGACAAGCTTATGGAGCTAGCCTCGGCTCGTCTC) and oligo 43 (GCGGCCGCGTCGACGGTATCGATAAGGTTGATATC) as primers (the *Hind*III and *Not*I sites are underlined) and pSK-H458 (Lin *et al.*, 1995) as the template. The 1.6 kb product was subcloned into pCRII to yield pCRII-Adeno-H458. The DNA 15 fragment coding for CAM1-H458 was isolated from pCRII-Adeno-H458 by digestion with *Hind*III and *Not*I, and the fragment was inserted into the adenoviral shuttle vector XCMV at the *Hind*III-*Not*I site to generate XCMV-CAM1-H458. AdCAM-H458 was generated by co-transfection as described above.

To construct the AdCAM1-G454 virus, a kinased *Hind*III recognition sequence was inserted at the *Sma*I site of pSK-IL60, which contains the sequence for 20 CAM1-G454 (also called C-CAM3) (Cheung *et al.*, 1993c). The 1.5 kb CAM1-G454 cDNA fragment was excised from the plasmid with *Hind*III and *Xmn*I and inserted into pBluescript pBSK (Stratagene, La Jolla, CA) to generate pSK-IL60-Adeno. A 1.5 kb *Hind*III-*Not*I fragment containing the CAM1-G454 cDNA, which lacks the C-terminal 65 amino acids of C-CAM1, was isolated from pSK-IL60-Adeno and 25 inserted into the adenovirus shuttle vector XCMV at the *Hind*III-*Not*I site to generate XCMV-CAM1-G454. AdCAM-G454 was generated by co-transfection as described above.

2. *Detection of C-CAM protein by FACS analysis.*

MDA-MB-468 cells were infected with recombinant adenovirus at various virus to cell (V/C) ratios. After 24 hr, fluorescence staining of C-CAM expressing cells was performed with an anti-C-CAM polyclonal antibody (Ab669) and 5 fluorescein isothiocyanate-conjugated secondary antibodies as described previously (Cheung *et al.*, 1993a). FACS analysis was performed as described previously (Hsieh *et al.*, 1995).

3. *Immunoprecipitation and western blotting.*

10 To analyze the C-CAM1 proteins expressed in MDA-MB-468 cells, aliquots of cell lysate from infected and uninfected cells were boiled in sodium dodecyl sulfate (SDS) sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Western immunoblotting with Ab669 was performed as previously described (Lin *et al.*, 1995). To immunoprecipitate the C-CAM1 proteins expressed 15 in MDA-MB-468 cells, aliquots of cell lysate (50 µl) from infected MDA-MB-468 cells were mixed with an equal volume of denaturing buffer (1 mM EDTA, 1% SDS, and 1 mM dithiothreitol, pH 8.0) and boiled for 5 min. After removal of insoluble material by centrifugation, the supernatants were diluted with immunoprecipitation buffer (150 mM NaCl, 1 mM EDTA, 0.5% deoxycholate, 1% Nonidet P-40, and 25 20 25 mM Tris-HCl, pH 7.5) to a final volume of 1 ml and incubated with 3 µl of antipeptide antibody and 20 µl of protein G-Sepharose (Pharmacia Inc., Piscataway, NJ) for 16 hr at 4°C with constant mixing. The immunoprecipitates were washed once with 0.8 ml of immunoprecipitation buffer containing 0.1 ml of saturated NaCl, then with 0.8 ml of immunoprecipitation buffer containing 0.1% SDS, and finally 25 with 0.8 ml of immunoprecipitation buffer alone. The materials bound to protein G-Sepharose were eluted by boiling the protein G-Sepharose in SDS sample buffer. Western immunoblotting with Ab669 was performed as previously described (Lin *et al.*, 1995).

4. Tumorigenicity in nude mice

MDA-MB-468 cells were infected with recombinant adenovirus at V/C ratio of 10:1. Twenty-four hr after infection, the cells were removed from the plates by trypsin digestion and washed twice with phosphate-buffered saline. One million cells 5 were then injected subcutaneously into each of six sites in the left and right flanks of nude mouse and tumor growth was monitored weekly. Tumor volume was estimated by the method of Rockwell *et al.* (1972).

B. Results

10 To study the effects of C-CAM1 expression on the *in vivo* tumorigenicity of the breast cancer cell line MDA-MB-468, a full-length C-CAM1 cDNA was inserted into an adenoviral vector to generate a C-CAM1 recombinant adenovirus (AdCAM1) as a gene transfer vehicle. The breast-tumor cell line MDA-MB-468 was highly tumorigenic when injected into nude mice, displaying tumor growth in 16/18 animals, 15 or 89% (Table 7). MDA-MB-468 cells infected with recombinant wild-type or mutant adenoviruses displayed varying incidences of tumor growth: AdCAM1, 2/18 (11%); AdCAM1-AS, 12/18 (67%); AdCAM1-ΔD1, 3/18 (17%), AdCAM1-H458, 14/18 (78%); AdCAM1-G454, 15/18 (83%) (Table 7). On western immunoblots with a polyclonal antibody against C-CAM (Ab669), C-CAM protein expression was not 20 detectable in MDA-MB-468 cells. This result is consistent with those observed in hepatoma and prostate cancer in that C-CAM is down-regulated in these tumors. To determine the expression efficiency of C-CAM1 in MDA-MB-468 cells, the inventors infected them with AdCAM1 at various virus to cell (V/C) ratios. Because C-CAM1 is an integral transmembrane protein, the efficiency of C-CAM expression was 25 determined by fluorescence-activated cell sorting (FACS) with a polyclonal antibody against C-CAM1 (Ab669) (Kleinerman *et al.*, 1995). About 45%, 56%, 69%, 73% and 86% of cells expressed C-CAM1 on their surfaces when infected with AdCAM1 at the V/C ratios of 5:1, 10:1, 20:1, 30:1, and 50:1, respectively, for 24 hr. In addition, the total amounts of protein expressed, as reflected by the increase in

fluorescence intensity, also increased as the V/C ratio increased. These results suggest that MDA-MB-468 cells are susceptible to adenoviral infection and can efficiently express and process the protein product of the C-CAM1 recombinant adenovirus.

5

Table 7. Tumor incidence induced by C-CAM1 and C-CAM1 mutant recombinant adenovirus infection of MDA-MB-468 cells

	<u>Adenovirus Infection</u>	<u>Tumor Incidence (%)</u>
10	Control (no infection)	16/18 (89)
	AdCAM1-AS	12/18 (67)
	AdCAM1	2/18 (11)
15	AdCAM1-ΔD1	3/18 (17)
	AdCAM1-H458	14/18 (78)
	AdCAM1-G454	15/18 (83)

Tumors larger than 10mm³ were considered to be positive.

20 Although the size of C-CAM1 is predicted to be 53 kDa based on its cDNA sequence (Lin and Guidotti, 1989; Lin *et al.*, 1991), the proteins expressed from MDA-MB-468 cells after infection with AdC-CAM1 had a relative mass of 120 kDa, suggesting that the C-CAM1 proteins were glycosylated. In addition to showing the 120 kDa protein, a western blot also showed an approximately 85 kDa protein. It is
 25 possible that the 85 kDa protein represents a C-CAM1 glycosylation variant, because western analysis of AdCAM1-infected cell lysate after treatment with peptide N-glycosidase F revealed only one protein band of about 53 kDa. Similar protein species were observed when the cells were infected with C-CAM1 mutant recombinant viruses. The AdCAM1-ΔD1 mutant proteins were about 20 kDa smaller
 30 than those of AdCAM1, perhaps because the first Ig domain contains three potential N-linked glycosylation sites (Lin *et al.*, 1991), and because the 102 amino acids coding for the first Ig domain were deleted. On the other hand, deletions in the

cytoplasmic domain (in AdCAM1-H458 and AdCAM1-H454) decreased the masses of the proteins by only about ~7 kDa. The identities of the mutant proteins were further confirmed by immunoprecipitation with antipeptide antibody against the cytoplasmic domain (anti-C3) (Lin *et al.*, 1991). AdCAM1 and AdCAM1-ΔD1 5 proteins were immunoprecipitated with antibody anti-C3, but AdCAM1-H458 and AdCAM1-G454 proteins were not, consistent with the deletion of the cytoplasmic domain.

EXAMPLE IX - Cell adhesion assay of recombinant adenovirus infected MDA-10 MB-468 cells.

This example shows the cell-adhesive activity of breast cancer cells infected with recombinant adenoviruses containing a wild type or mutant C-CAM1 cDNA.

A. Materials and Methods

1. Cell adhesion assay

15 MDA-MB-468 cells were infected with AdCAM1 or its mutants. Twenty-four hr after infection, the cells were trypsinized from the tissue culture plates and resuspended in phosphate buffered saline. These cell suspensions (1 ml) were mixed gently at room temperature to allow the formation of cell aggregates. The number of single cells remaining in suspension was determined with a hemacytometer after 5 hr. 20 The extent of cell aggregation was expressed as the decrease in the percentages of single cells.

B. Results

To test the cell-adhesive activity of these various mutants, cell-aggregation assays were performed. MDA-MB-468 cells were infected with recombinant adenovirus at V/C ratio of 10:1. At 24 hr after infection, the cells were trypsinized, and their ability to form aggregates was monitored. As shown in FIG. 13, MDA-MB-468 cells expressing wild-type C-CAM1 protein were able to aggregate, as evidenced by the decrease in the percentage of single cells (about 50%), as compared with cells

infected with control antisense adenovirus. Expression of AdCAM1-ΔD1 did not confer cell adhesion (FIG. 13), which is consistent with the inventors' observations in the insect cell system (Cheung *et al.*, 1993a). Both AdCAM1-H458 and AdCAM1-G454 were able to cause MDA-MB-468 cells to aggregate. These results are in 5 contrast to those of the inventors' insect cell studies, in which AdCAM1-G454 had no cell-adhesion activity (Cheung *et al.*, 1993c). These differences may be a consequence of the different cell types used for expression or the amount of protein expressed per cell.

10 **EXAMPLE X - C-CAM1 cytoplasmic domain involved in tumor suppression**

This example shows the effects of expression of C-CAM1 mutants on the *in vivo* tumorigenicity of MDA-MB-468 cells.

A. Materials and Methods

1. *Tumorigenicity in nude mice*

15 MDA-MB-468 cells were infected with recombinant adenovirus at V/C ratio of 10:1. Twenty-four hr after infection, the cells were removed from the plates by trypsin digestion and washed twice with phosphate-buffered saline. One million cells were then injected subcutaneously into each of six sites in the left and right flanks of nude mouse and tumor growth was monitored weekly. Tumor volume was estimated 20 by the method of Rockwell *et al.* (1972).

B. Results

The tumor-suppressive activities of the C-CAM1 mutants were studied by infecting MDA-MB-468 cells with the recombinant adenoviruses at a V/C ratio of 25 10:1. The efficiency of infection was determined by flow cytometry at 24 hr after infection. About 45% of the cells infected with AdCAM1, AdCAM1-ΔD1, or AdCAM1-H458, were positive for C-CAM staining. About 80% of the cells infected with AdCAM1-G454 were positive for C-CAM staining. The reason for the higher efficiency of AdCAM1-G454 expression is not clear. The infected cells were then

injected subcutaneously into nude mice, and tumor growth was monitored weekly. AdCAM1-ΔD1 infection reduced both the tumor incidence and size in a fashion similar to that of infection with AdCAM1 (Table 7 and FIG. 12 A and B). These results suggest that the adhesion activity of C-CAM1 is not required for C-CAM1-mediated tumor suppression. On the other hand, C-CAM1 mutants with deletions in their cytoplasmic domains (AdCAM1-H458 and AdCAM1-G454) did not suppress tumorigenicity, producing a tumor incidence and volume similar to those of controls (Table 7 and FIG. 12 A and B). These results strongly suggest that the cytoplasmic domain of C-CAM1 is involved in C-CAM1-mediated tumor suppression.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated
5 herein by reference.

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25

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(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

35

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10 (2) INFORMATION FOR SEQ ID NO:1:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1921 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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10	CGCTGTCCA	CCCAACGTTG	TGAGGGAGAA	GAGGGTTCTT	TTACTCGCTC	ACAATCTGCC	240
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20	TGAGGCCCTTC	GTATCATTAA	TGTGTGAAACC	GTACACTAAAC	AATAACAGCT	ACCTGTGGAG	600
	CAGAAATGGT	GAAGGCCTCT	CAGAAGGTGA	CAGGGTGA	TTCTCTGAGG	GCAACAGGAC	660
25	TCTCACTTTA	CTCAACGTCA	GGAGGACTGTG	CAAAGGATAAC	TATGAGTGTG	AAGCCGGAA	720
	CCCAGGGACT	TTCAATCGAA	GTGACCCATT	CAACCTGGAC	GTATCTATG	GTCCGGACGC	780
	CCCAGTTATA	TCCCTCCAG	ATATTTATT	GCATCAAGGG	TCAAACCTCA	ACCTCTCCTG	840
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10	AGACCCATT AGAGAGAG ATGCCGGGA CTATCAGTGT GAGATCTCCA ACCGGTCA	1260
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15	AATAGCAGCG CTGGCATACT TCCTTTATT CAGGAAGACT GGCGGGGAA GTGACCATCG	1440
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	TCCTAACAG GTGGATGACG TCTCATACT TGTCCTGAAC TTCAATGCC AGCAATCCAA	1560
20	ACGACCAACT TCAAGCTCTT CAAGCCCCAC AGAAACAGTT TATTCGGTAG TCAAAAGAA	1620
	GTGACATTGT CTGTCCTGCT GACTGACCA GTGATGCATT CCAGATTCTC TGTCACGGAG	1680
25	AGACCCCTGCA CACAGGCAC GTGCGCGTGC ACACACACAC ACACACAC ACACATGCAC	1740
	ACACACACGC ACACACGCAC GCACACACAC ACATGCACAC ACGGCACAC ACATGTATAC	1800
30	ACTCTATTGA CTCCCTCCGT GTTAATGAA GAAAAGGTG ATCCCTGGAGC CTACAGAAA	1860

- 109 -

CCCCAACCCCTT TCTAGGCATT AAAATTTGGC CGAGGAGAG GAACCAAAAC GGCCACCCC

1920

G

1921

- 110 -

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- 5
(A) LENGTH: 519 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

10
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Leu Ala Ser Ala Arg Leu Leu Arg Gly Gln Ile Pro Trp Arg
1 5 10 15

Gly Leu Leu Leu Thr Ala Ser Leu Leu Thr Tyr Trp Ser Pro Leu Thr
15 20 25 30

Thr Ala Gln Val Thr Val Asp Ala Val Pro Pro Asn Val Val Glu Glu
20 35 40 45

Lys Ser Val Leu Leu Leu Ala His Asn Leu Pro Gln Glu Phe Gln Val
50 55 60

Phe Tyr Trp Tyr Lys Gly Thr Thr Leu Asn Pro Asp Ser Glu Ile Ala
25 65 70 75 80

Arg Tyr Ile Arg Ser Asp Asn Met Ser Lys Thr Gly Pro Ala Tyr Ser
85 90 95

Gly Arg Glu Thr Ile Tyr Ser Asn Gly Ser Leu Phe Phe Gln Asn Val
30 100 105 110

Asn Lys Thr Asp Glu Arg Ala Tyr Thr Leu Ser Val Phe Asp Gln Gln
35 115 120 125

Phe Asn Pro Ile Gln Thr Ser Val Gln Phe Arg Val Tyr Pro Ala Leu
130 135 140

Gln Lys Pro Asn Val Thr Gly Asn Asn Ser Asn Pro Met Glu Gly Glu
40 145 150 155 160

Pro Phe Val Ser Leu Met Cys Glu Pro Tyr Thr Asn Asn Thr Ser Tyr
165 170 175

Leu Trp Ser Arg Asn Gly Glu Ser Leu Ser Glu Gly Asp Arg Val Thr
45 180 185 190

Phe Ser Glu Gly Asn Arg Thr Leu Thr Leu Leu Asn Val Arg Arg Thr
50 195 200 205

Asp Lys Gly Tyr Tyr Glu Cys Glu Ala Arg Asn Pro Ala Thr Phe Asn
210 215 220

- 111 -

	Arg Ser Asp Pro Phe Asn Leu Asp Val Ile Tyr Gly Pro Asp Ala Pro			
225	230	235	240	
5	Val Ile Ser Pro Pro Asp Ile Tyr Leu His Gln Gly Ser Asn Leu Asn			
	245	250	255	
	Leu Ser Cys His Ala Asp Ser Asn Pro Pro Ala Gln Tyr Phe Trp Leu			
	260	265	270	
10	Ile Asn Glu Lys Leu Gln Thr Ser Ser Gln Glu Leu Phe Ile Ser Asn			
	275	280	285	
15	Ile Thr Thr Asn Asn Ser Gly Thr Tyr Ala Cys Phe Val Asn Asn Thr			
	290	295	300	
	Val Thr Gly Leu Ser Arg Thr Thr Val Lys Asn Ile Thr Val Phe Glu			
	305	310	315	320
20	Pro Val Thr Gln Pro Ser Ile Gln Ile Thr Asn Thr Thr Val Lys Glu			
	325	330	335	
	Leu Gly Ser Val Thr Leu Thr Cys Phe Ser Lys Asp Thr Gly Val Ser			
	340	345	350	
25	Val Arg Trp Leu Phe Asn Ser Gln Ser Leu Gln Leu Thr Asp Arg Met			
	355	360	365	
30	Thr Leu Ser Gln Asp Asn Ser Thr Leu Arg Ile Asp Pro Ile Lys Arg			
	370	375	380	
	Glu Asp Ala Gly Asp Tyr Gln Cys Glu Ile Ser Asn Pro Val Ser Phe			
	385	390	395	400
35	Arg Ile Ser His Pro Ile Lys Leu Asp Val Ile Pro Asp Pro Thr Gln			
	405	410	415	
	Gly Asn Ser Gly Leu Ser Glu Gly Ala Ile Ala Gly Ile Val Ile Gly			
	420	425	430	
40	Ser Val Ala Gly Val Ala Leu Ile Ala Ala Leu Ala Tyr Phe Leu Tyr			
	435	440	445	
	Ser Arg Lys Thr Gly Gly Ser Asp His Arg Asp Leu Thr Glu His			
45	450	455	460	
	Lys Pro Ser Thr Ser Ser His Asn Leu Gly Pro Ser Asp Asp Ser Pro			
	465	470	475	480
50	Asn Lys Val Asp Asp Val Ser Tyr Ser Val Leu Asn Phe Asn Ala Gln			
	485	490	495	

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Gln Ser Lys Arg Pro Thr Ser Ala Ser Ser Ser Pro Thr Glu Thr Val
500 505 510

Tyr Ser Val Val Lys Lys Lys
5 515

CLAIMS:

1. An expression construct comprising a promoter functional in
5 eukaryotic cells and a nucleic acid encoding a C-CAM, wherein said nucleic acid is
under transcriptional control of said promoter.

2. The expression construct according to claim 1, further comprising a
10 polyadenylation signal.

3. The expression construct according to claim 1, further comprising a
selectable marker.

15

4. The expression construct according to claim 1, wherein said nucleic
acid is a cDNA.

20

5. The expression construct according to claim 1, wherein said nucleic
acid is a genomic DNA.

25

6. The expression construct according to claim 1, wherein said expression
construct is an adenovirus.

7. The expression construct according to claim 6, wherein said adenovirus
30 lacks at least a portion of the E1 region.

8. The expression construct according to claim 1, wherein said nucleic acid is positioned in a sense orientation with respect to said promoter.

5

9. The expression construct according to claim 1, wherein said nucleic acid is positioned in an antisense orientation with respect to said promoter.

10

10. A pharmaceutical composition comprising (i) an expression construct comprising a promoter functional in eukaryotic cells and a nucleic acid encoding a C-CAM, wherein said nucleic acid is under transcriptional control of said promoter and (ii) a pharmaceutically acceptable buffer, solvent or diluent.

15

11. A method for restoring C-CAM function in a cell that lacks C-CAM function comprising the steps of:

20

providing an expression construct comprising a promoter functional in eukaryotic cells and a nucleic acid encoding a C-CAM, said nucleic acid under the control of said promoter and nucleic acid positioned in a sense orientation to said promoter; and

contacting said expression construct with said cell that lacks C-CAM function.

25

12. The method of claim 11, wherein said cell is a transformed cell and said contacting reverses said transformed phenotype.

30

13. The method of claim 12, wherein said cell is a prostate or tumor cell.

14. The method of claim 12, wherein said cell is a bladder tumor cell.

5

15. The method of claim 12, wherein said cell is a breast tumor cell.

16. The method of claim 11, wherein said expression construct is an
10 adenovirus.

17. A method for inhibiting C-CAM function in a cell comprising the steps
of:

15

providing an expression construct comprising a promoter functional in
eukaryotic cells and a nucleic acid encoding a C-CAM, said nucleic
acid under the control of said promoter and positioned in an antisense
orientation to said promoter; and

20

contacting said expression construct with said cell.

18. The method of claim 17, wherein said cell is transformed by said
contacting.

25

19. The method of claim 17, wherein said expression construct is an
adenovirus.

30

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20. A method of treating a mammal with cancer comprising:
providing a pharmaceutical composition comprising (a) an adenovirus
comprising a promoter functional in eukaryotic cells and a nucleic acid
5 encoding a C-CAM, said nucleic acid under the control of said
promoter and positioned in a sense orientation to said promoter, and
(b) a pharmaceutically acceptable buffer, solvent or diluent; and
administering said pharmaceutical composition to said mammal.

10

21. The method according to claim 20, wherein said mammal is a human.

15

22. The method according to claim 20, wherein said administering is via
intravenous injection.

20

23. The method according to claim 20, wherein said administering is via
orthotopic injection.

25

24. The method according to claim 20, wherein said cancer is prostate
cancer.

25. The method according to claim 20, wherein said cancer is bladder
cancer.

30

- 117 -

26. The method according to claim 20, wherein said cancer is breast cancer.

27. A kit comprising, in suitable container means, an expression construct comprising a promoter functional in eukaryotic cells and a nucleic acid encoding a C-CAM, wherein said nucleic acid is under transcriptional control of said promoter, and a pharmaceutically acceptable buffer, solvent or diluent.

10 28. A method for detecting cancer cells in a sample by detecting a nucleic acid encoding a C-CAM.

15 29. A method for detecting cancer cells in a sample by detecting a C-CAM.

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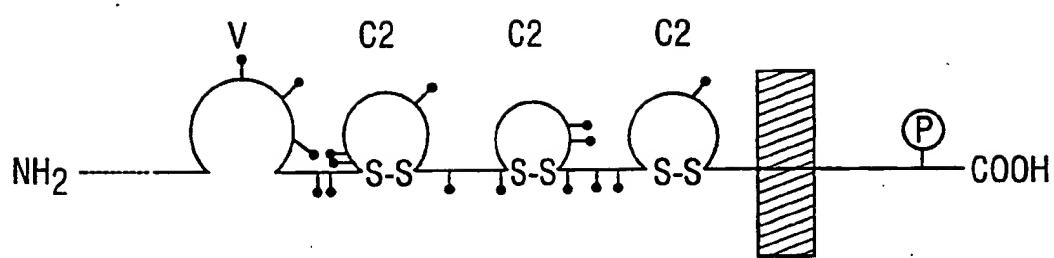


FIG. 1

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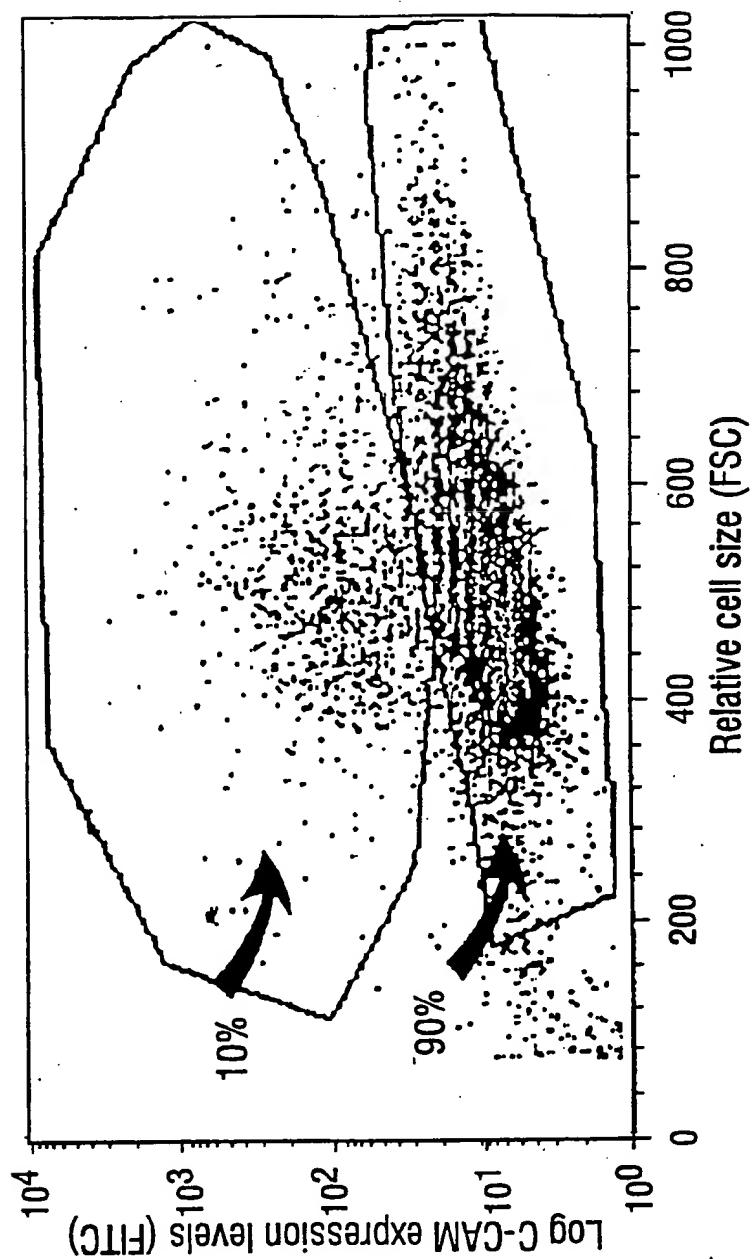


FIG. 2A

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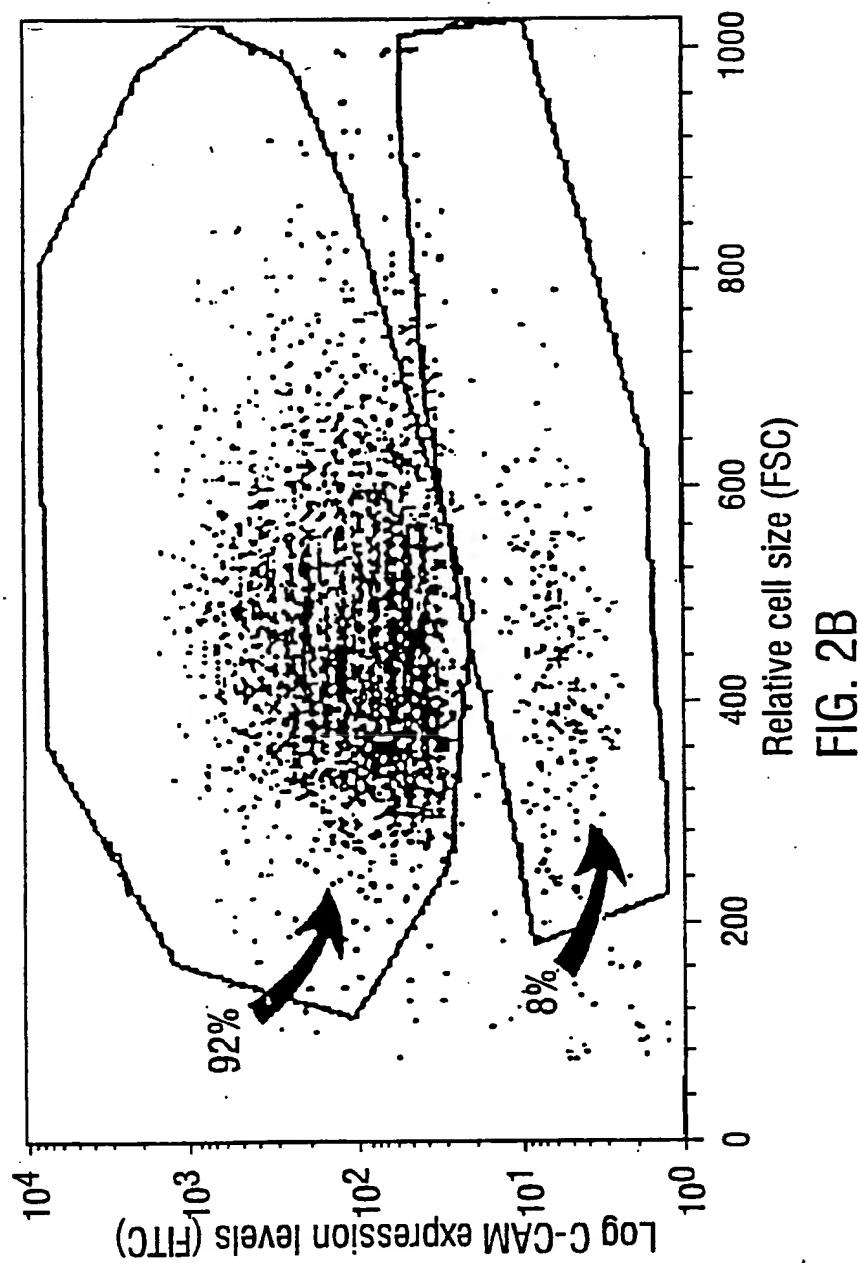


FIG. 2B

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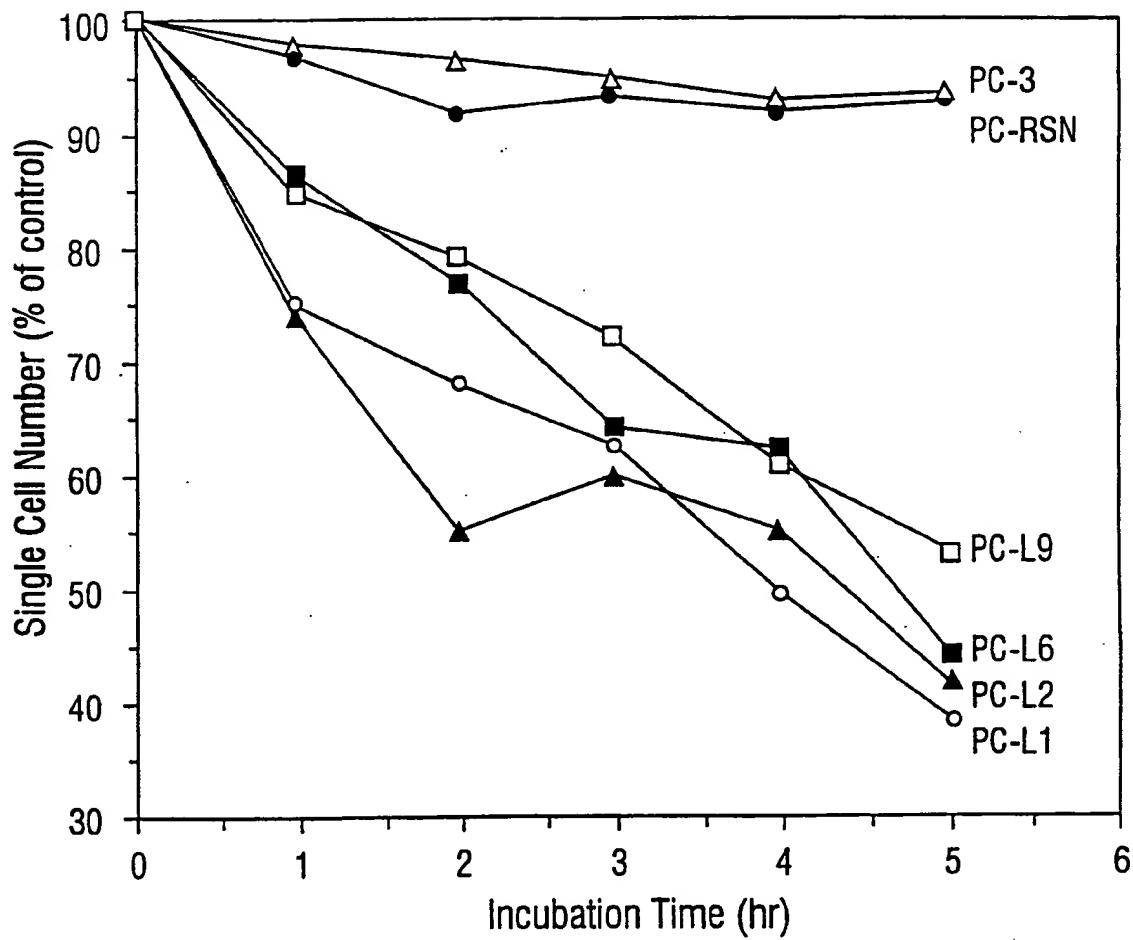


FIG. 3

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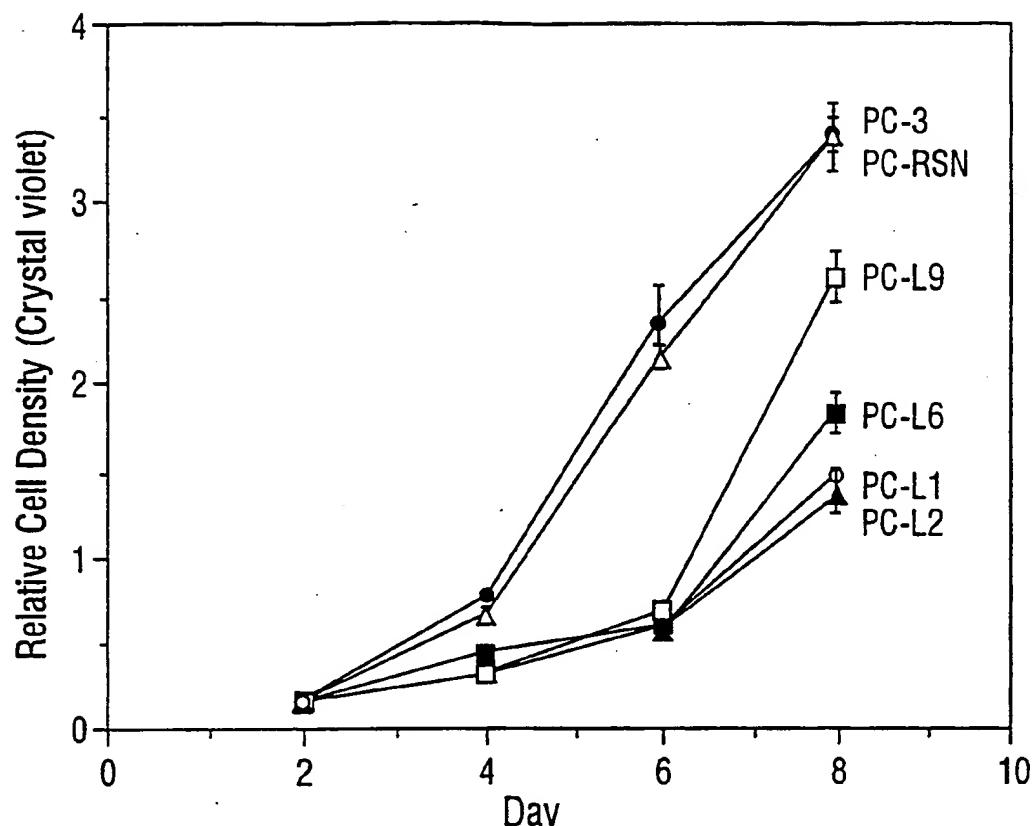


FIG. 4A

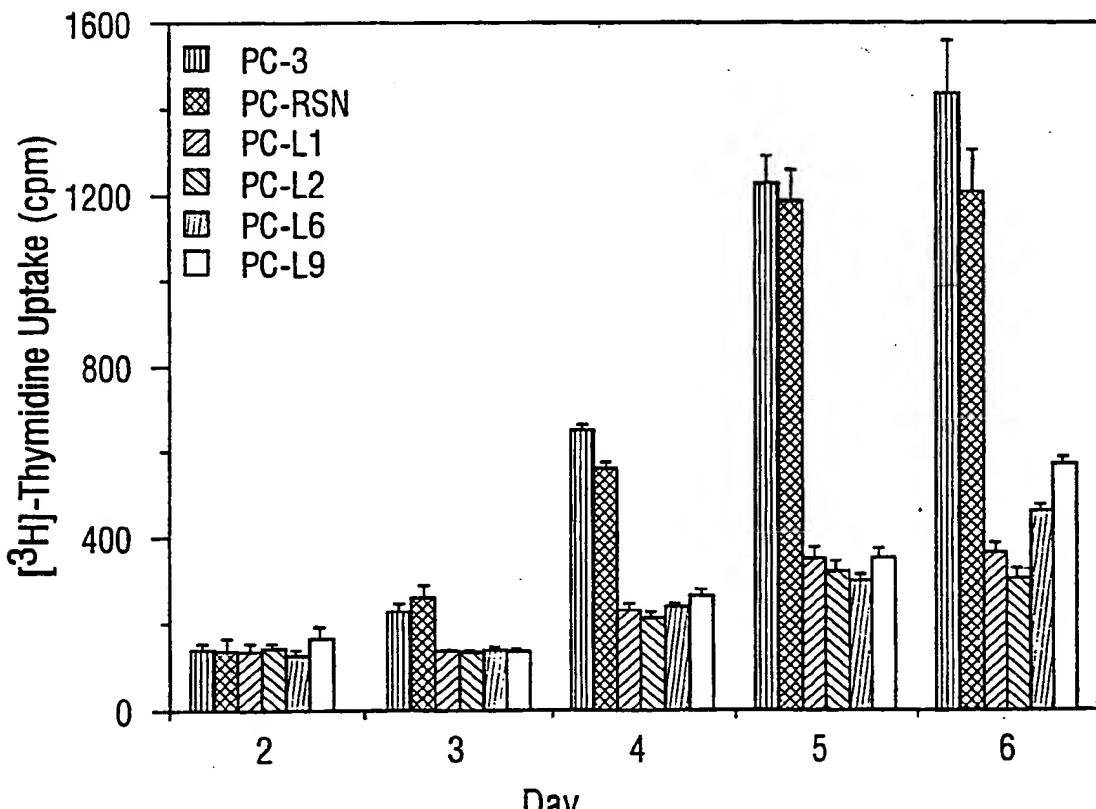


FIG. 4B

SUBSTITUTE SHEET (RULE 26)

6/19

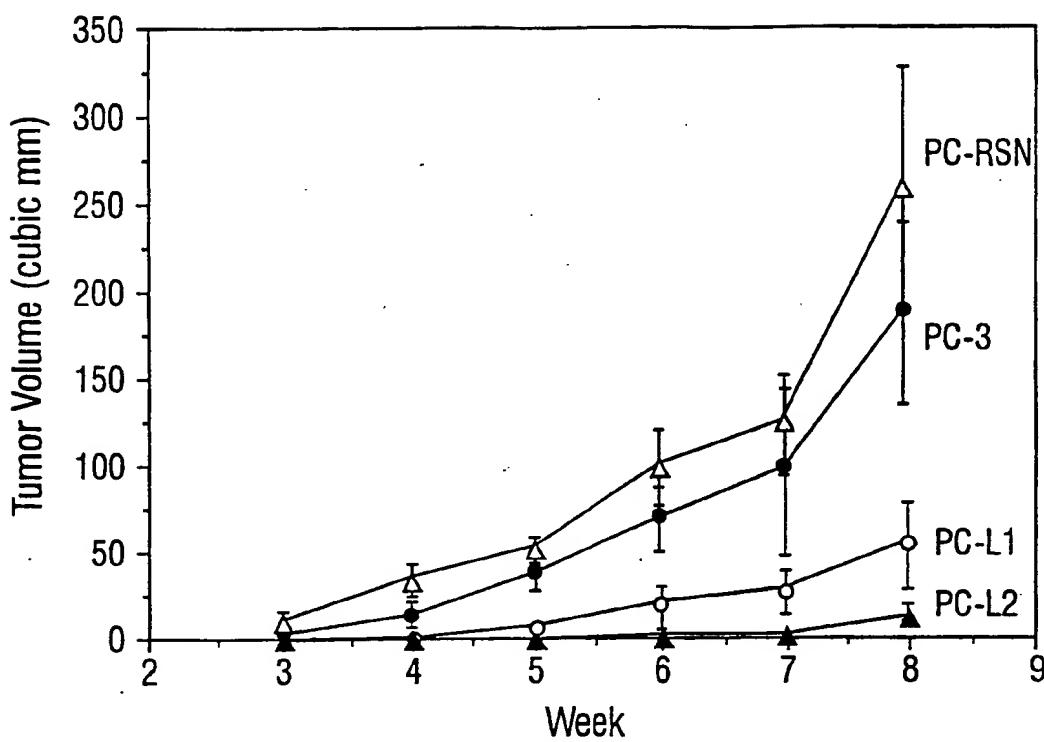
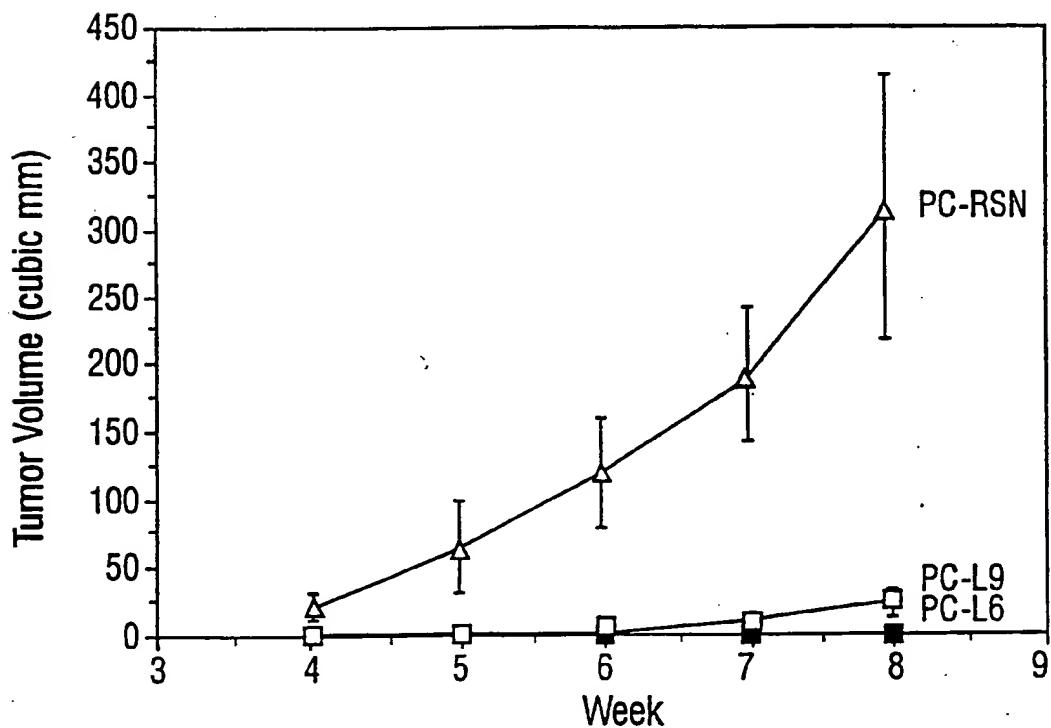


FIG. 5A

FIG. 5B
SUBSTITUTE SHEET (RULE 26)

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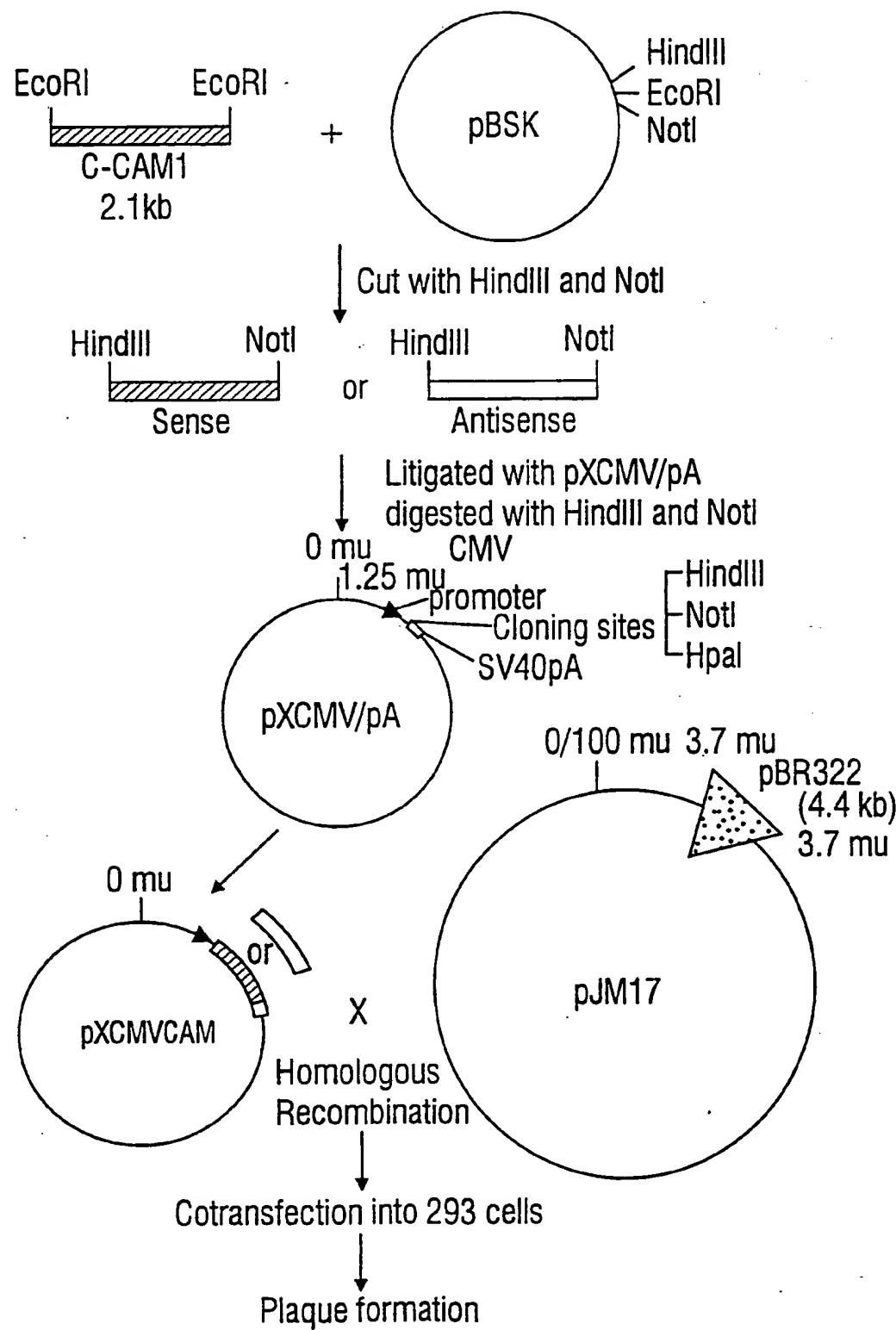


FIG. 6
SUBSTITUTE SHEET (RULE 26)

8/19

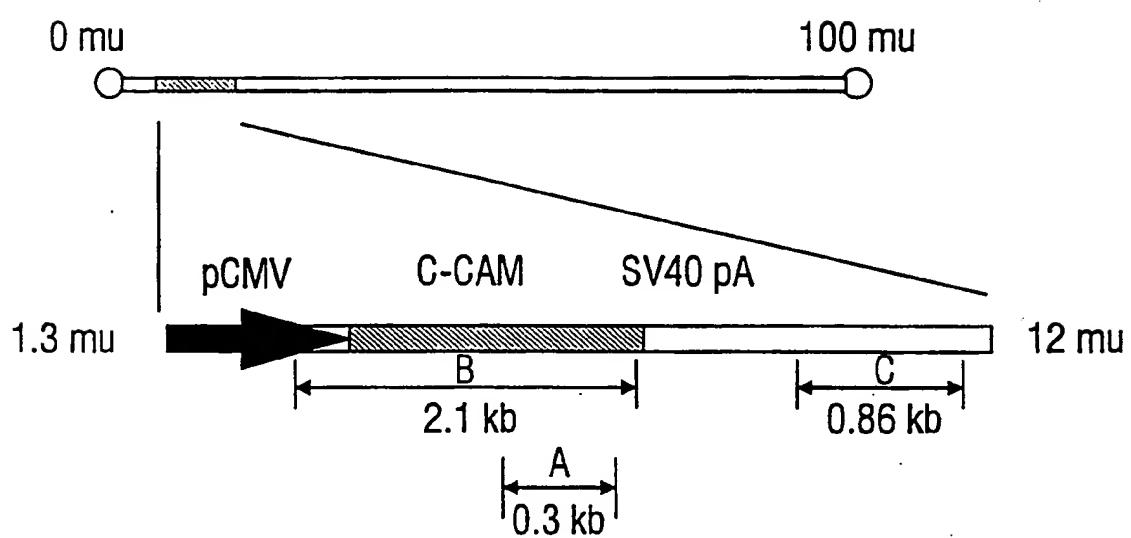


FIG. 7
SUBSTITUTE SHEET (RULE 26)

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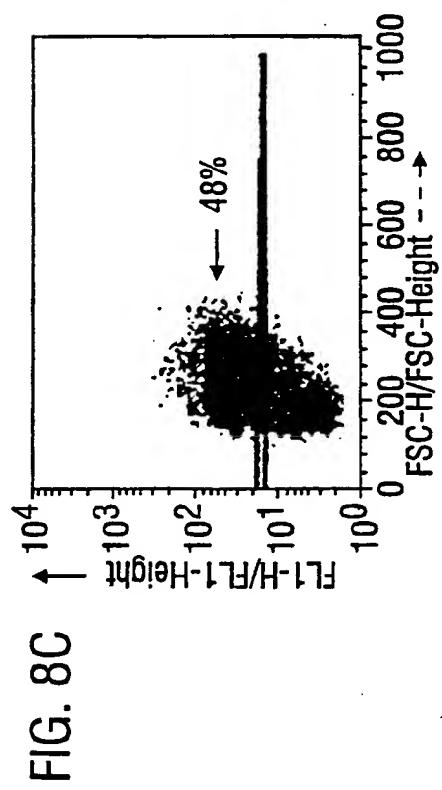
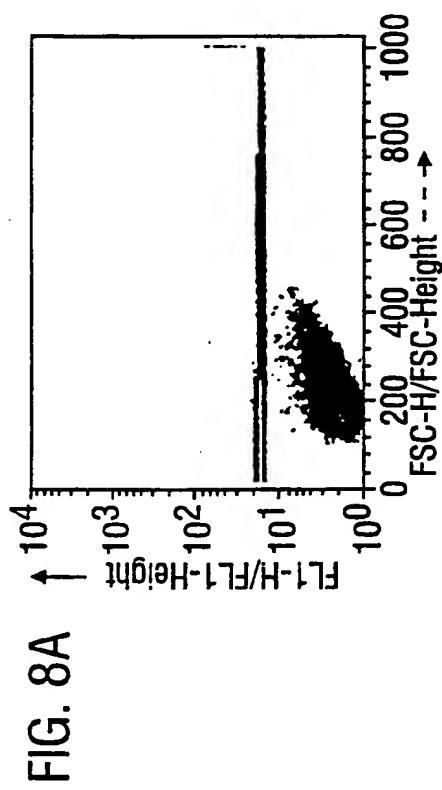


FIG. 8B

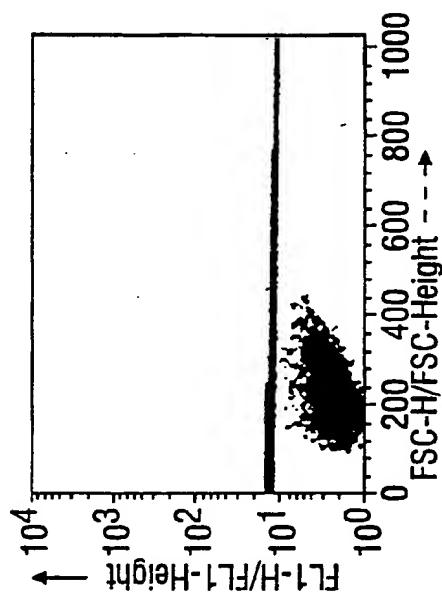
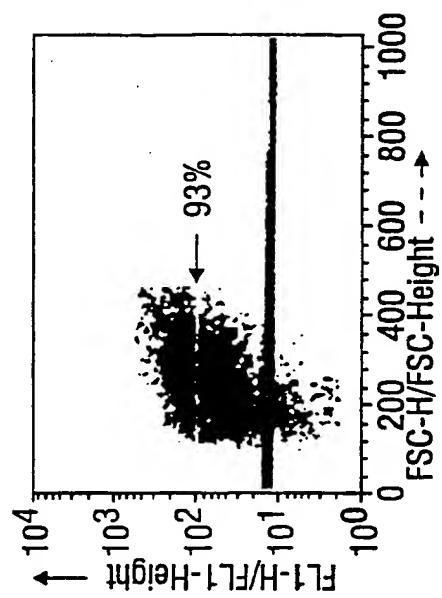


FIG. 8D



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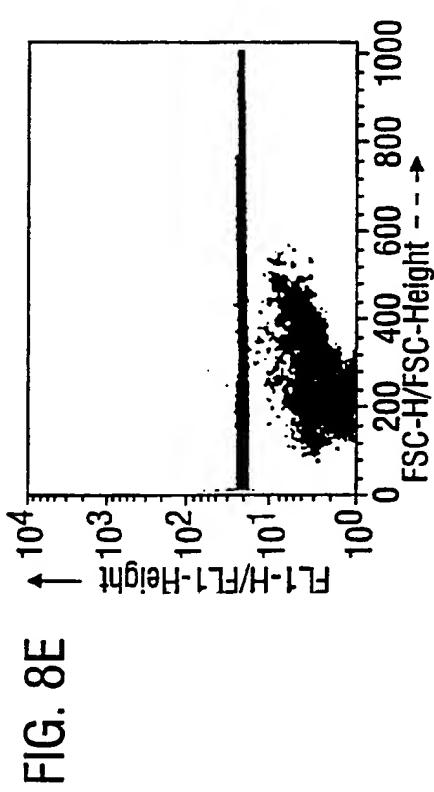


FIG. 8F

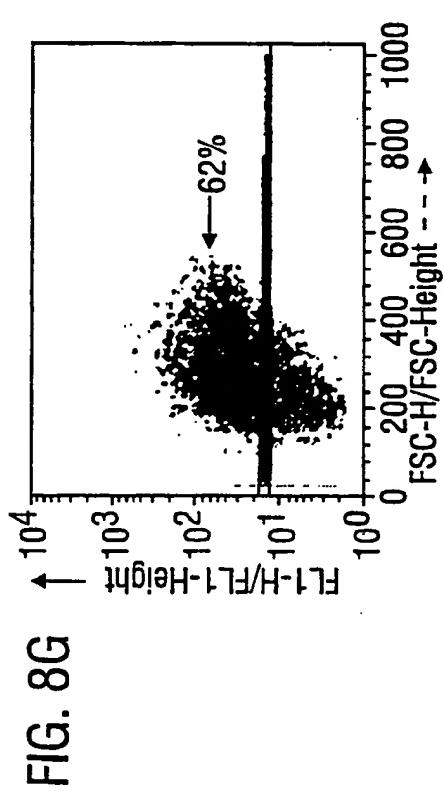
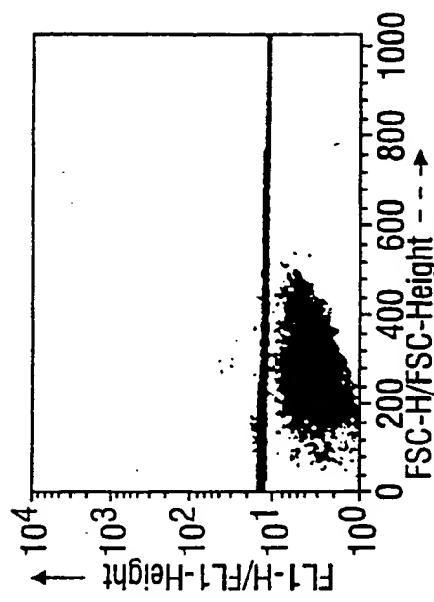
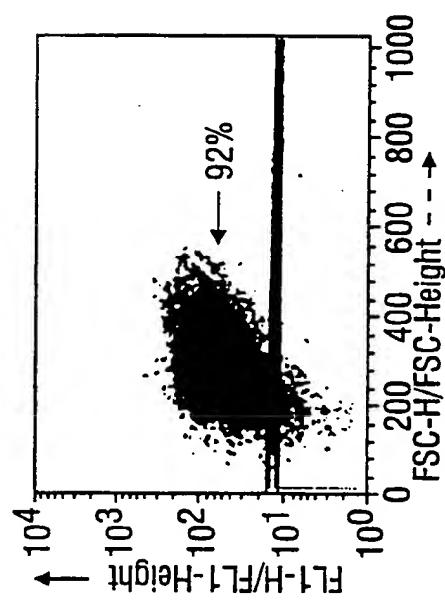


FIG. 8H



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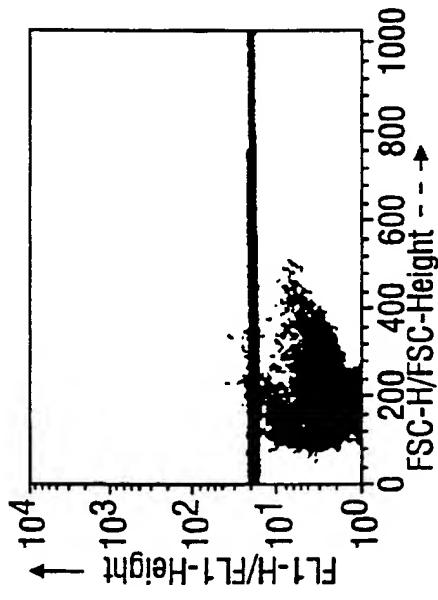


FIG. 8I

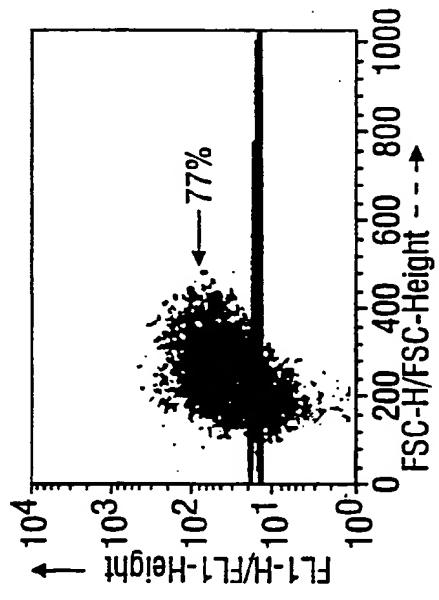


FIG. 8K

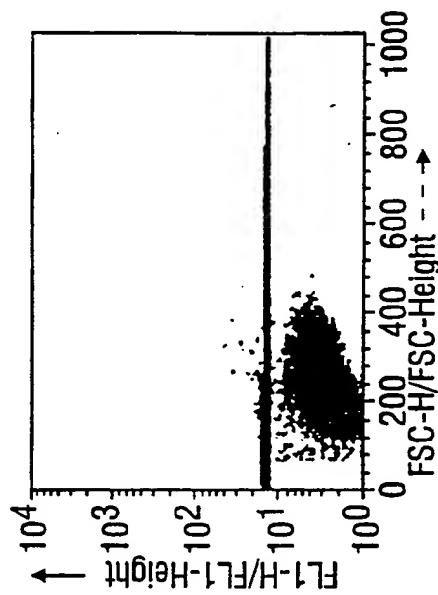


FIG. 8J

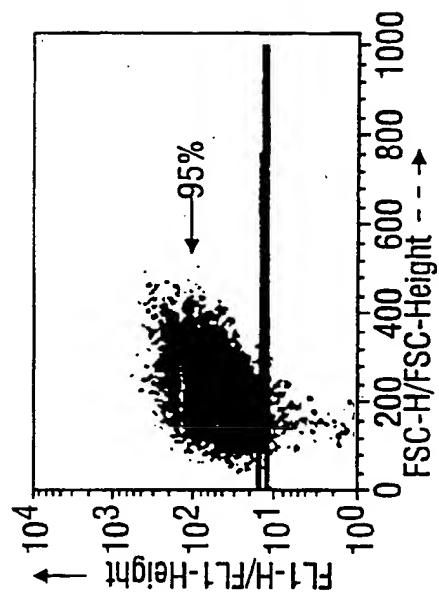


FIG. 8L

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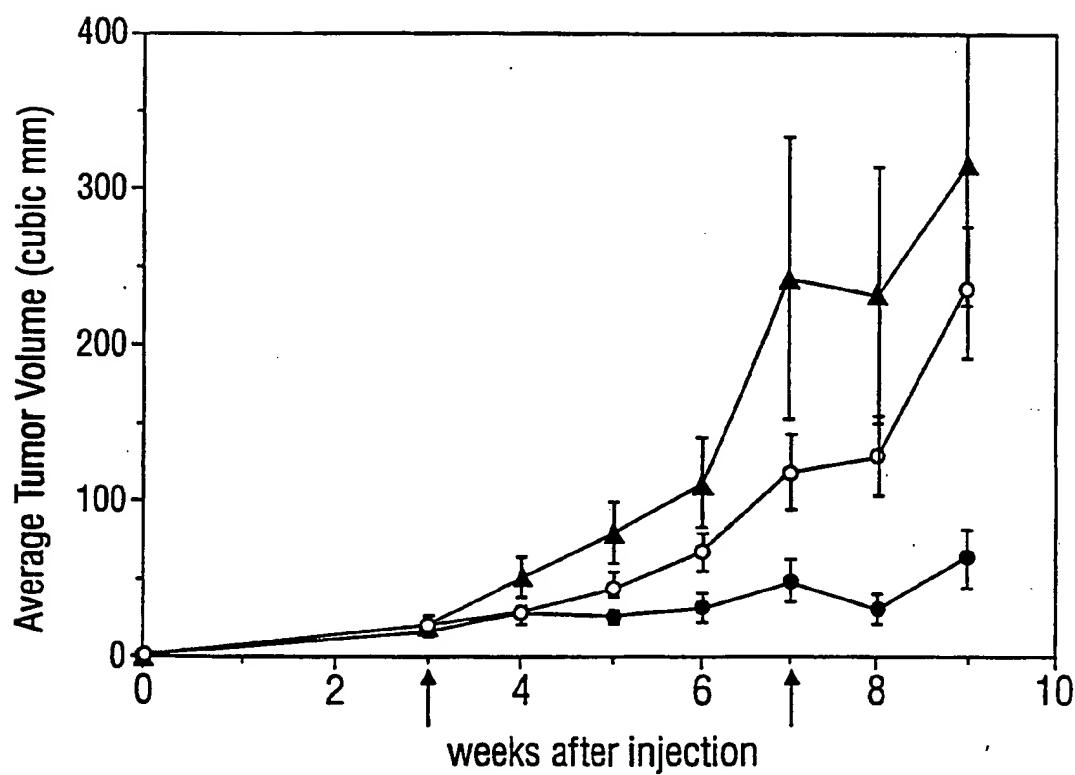


FIG. 9
SUBSTITUTE SHEET (RULE 26)

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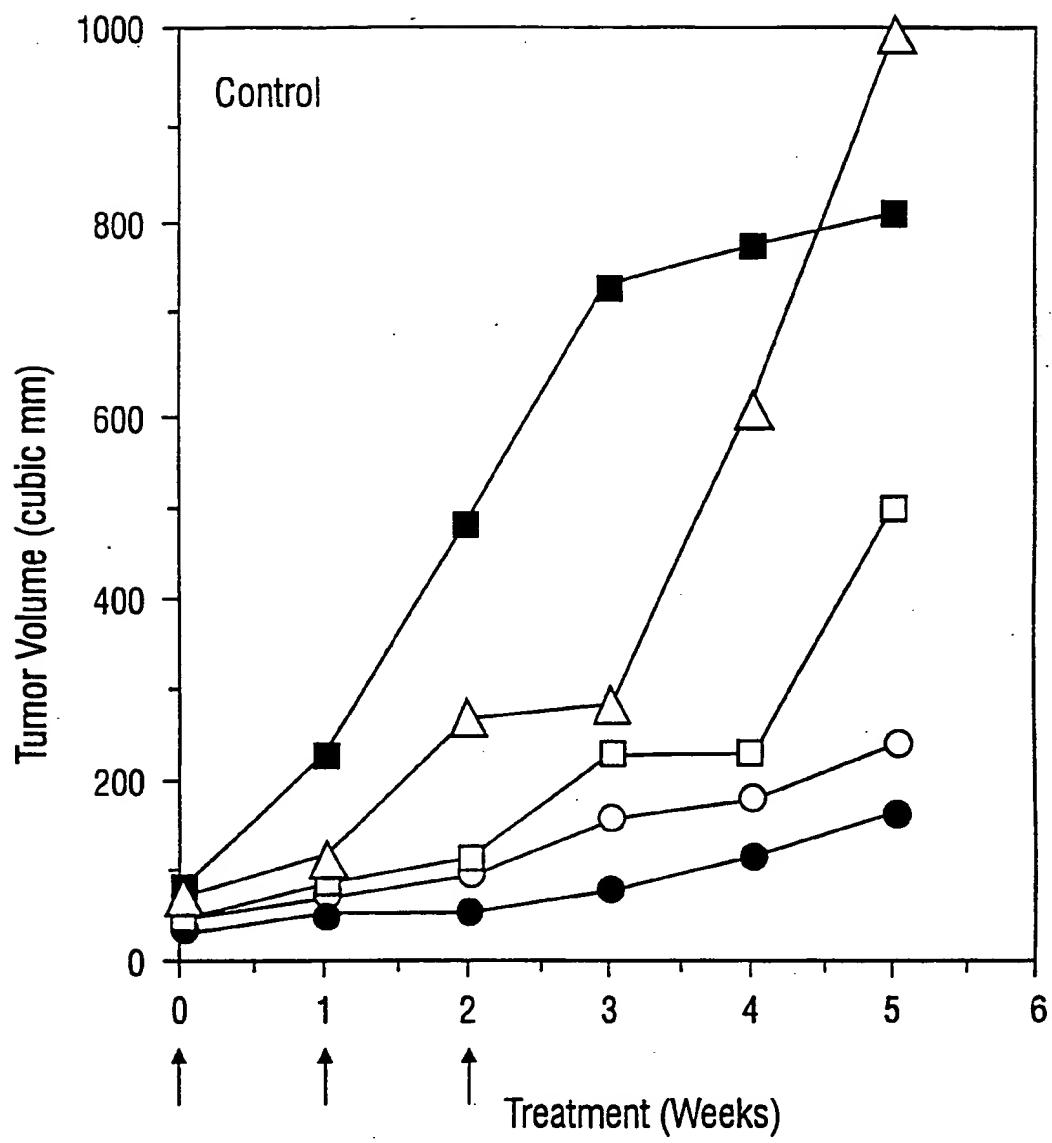


FIG. 10A

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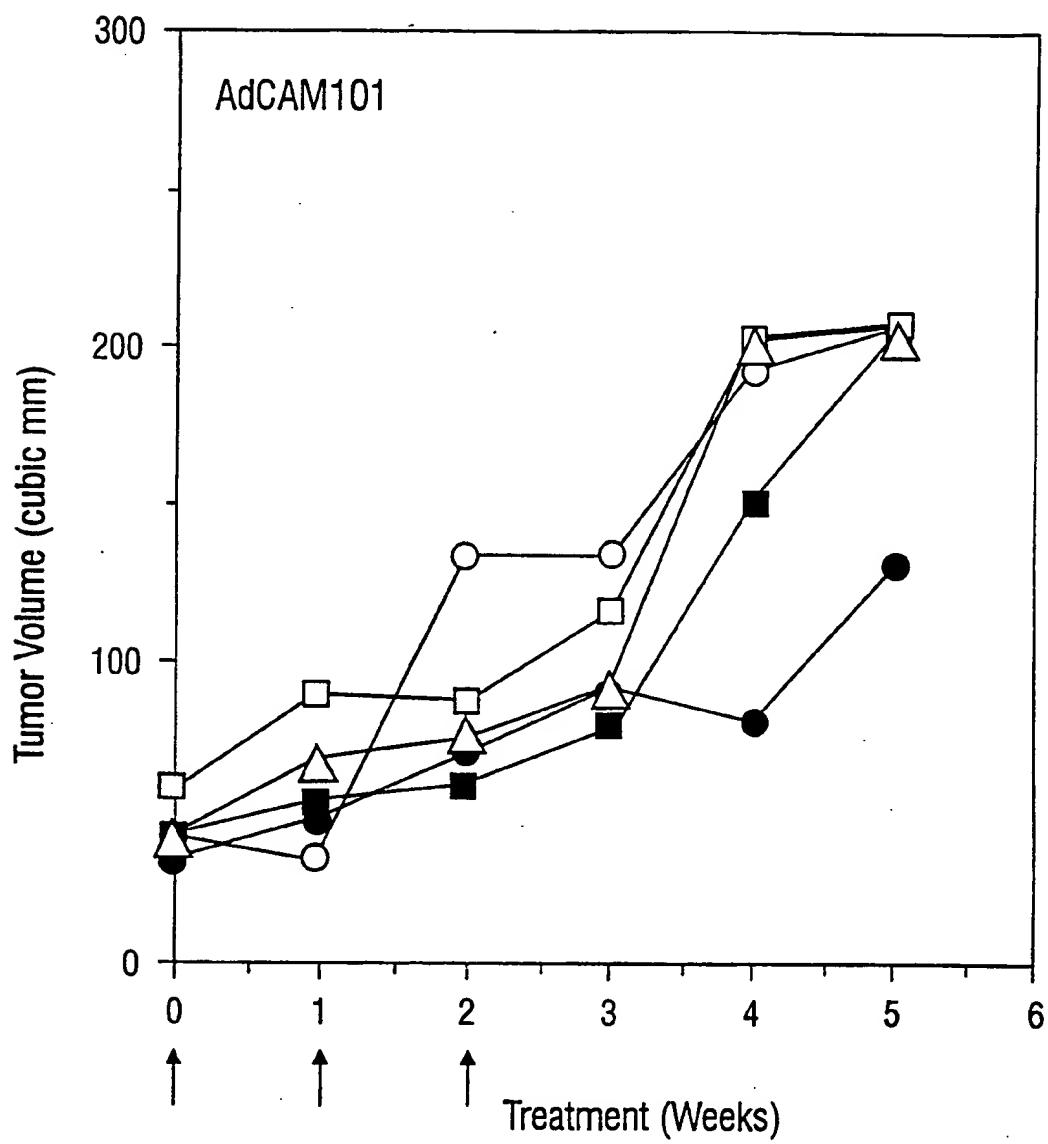


FIG. 10B

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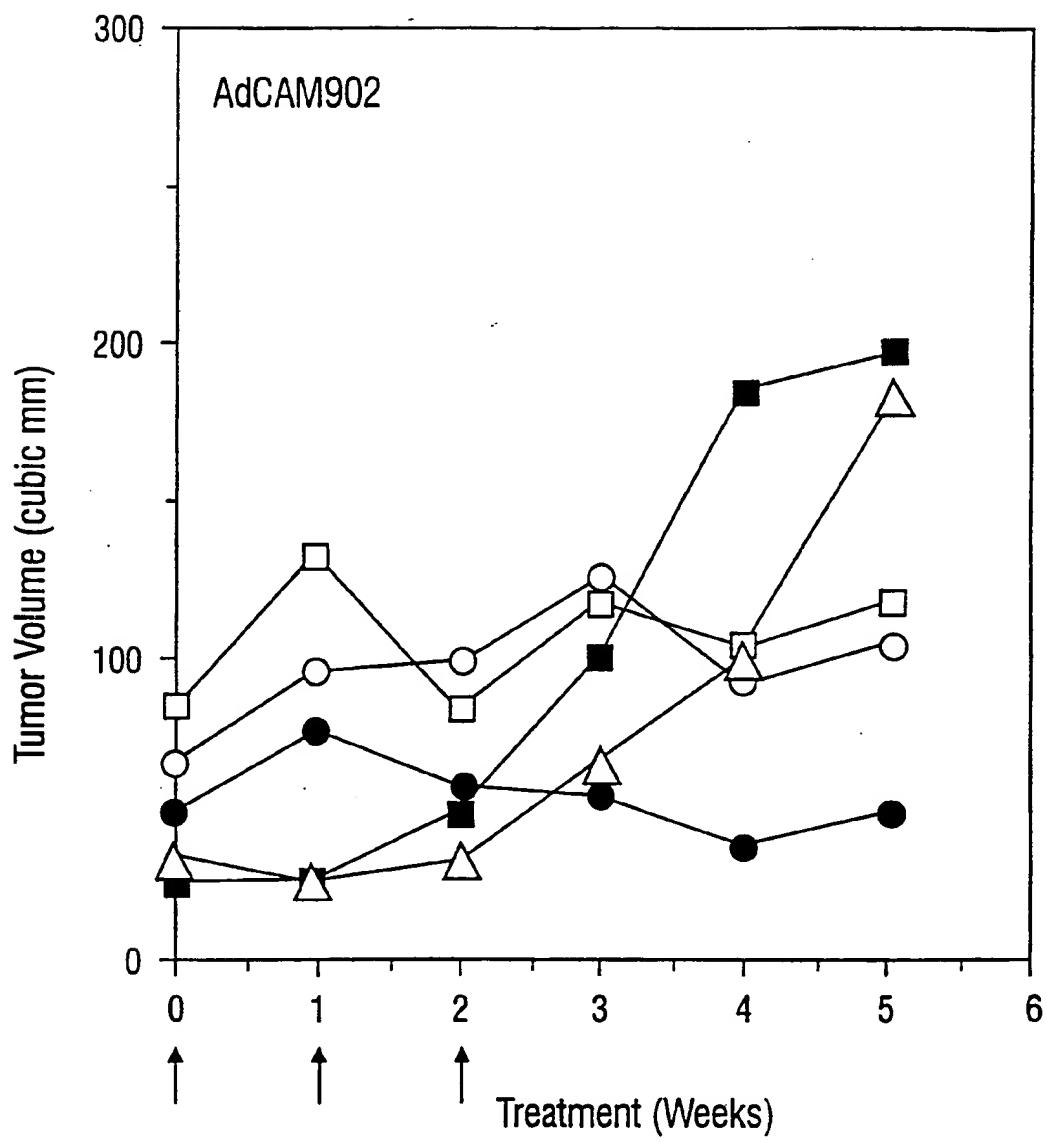


FIG. 10C

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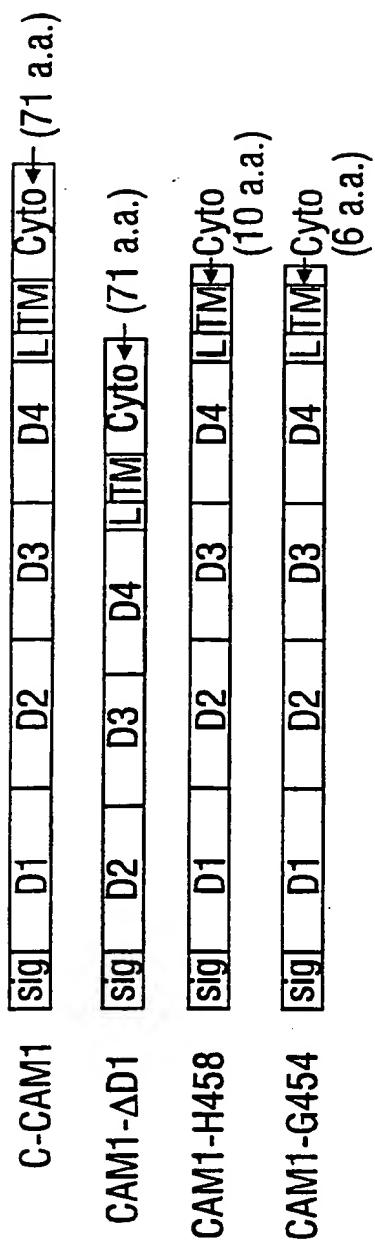


FIG. 11

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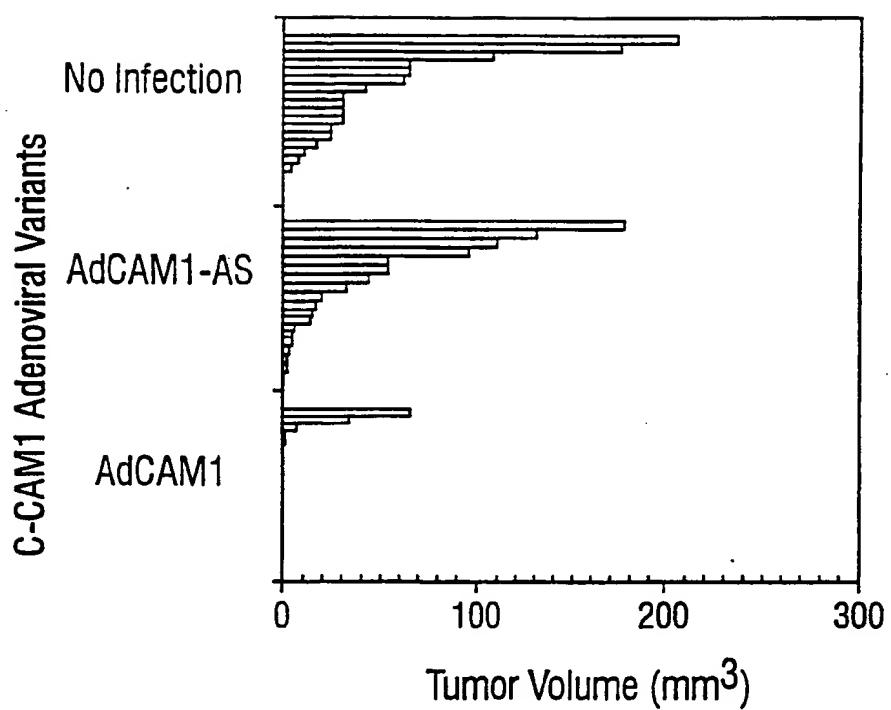


FIG. 12A

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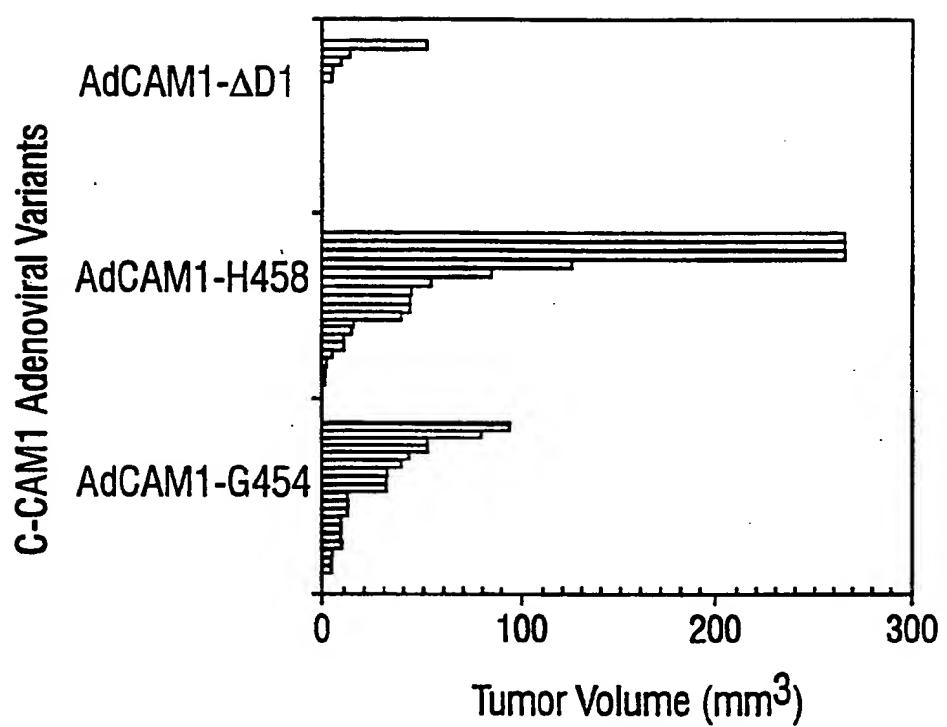


FIG. 12B

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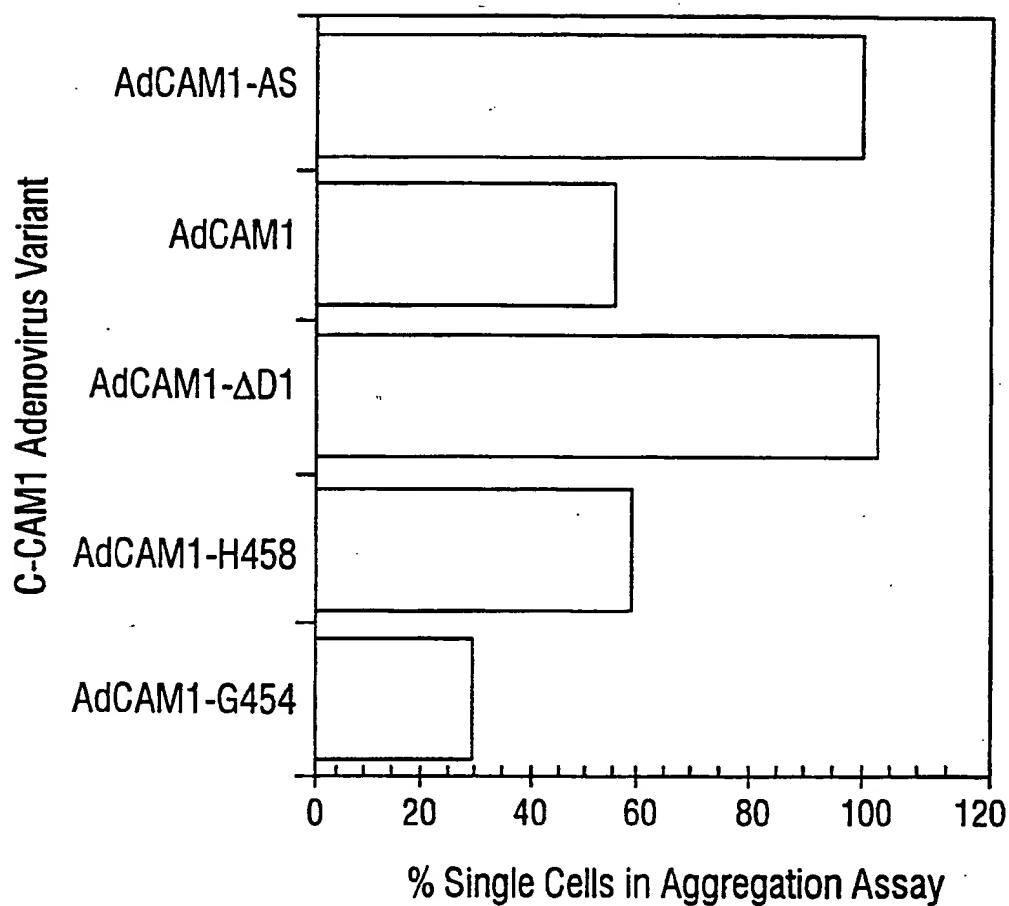


FIG. 13

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/10696

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/12 C07K14/705 C12N15/86 A61K48/00 G01N33/50
 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF UROLOGY, vol. 153, no. 4 sup, April 1995, page 306a XP000603637 KLEINERMAN, D.I. ET AL.: "C-CAM as a tumor suppressor in human prostate carcinoma- A potential target for gene therapy" see Abstract 309 ---	1,6, 8-13, 16-20, 24,27-29
X	JOURNAL OF UROLOGY, vol. 153, no. 4 sup, April 1995, page 522a XP000603639 KLEINERMAN, D.I. ET AL.: "The tumor-suppressor role of an epithelial cell-specific Cell Adhesion Molecule (C-CAM) in human bladder cancer progression" see Abstract 1175 ---	1,6, 8-12,14, 16,20, 23,25,27

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

3 October 1996

Date of mailing of the international search report

28.10.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
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Fax. (+31-70) 340-3016

Authorized officer

Chambonnet, F

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/10696

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FASEB JOURNAL FOR EXPERIMENTAL BIOLOGY, vol. 9, no. 3, 9 - 13 April 1995, BETHESDA, MD US. page a1 XP0002015051 COMEGRYS, M. ET AL.: "Rat strain and cell-type specific expression of C-CAM 2" see abstract & Experimental biology 95, Atlanta, USA, April 9-13 1995 Poster discussion	1,4,27
X	---	
X	JOURNAL OF BIOLOGICAL CHEMISTRY (MICROFILMS), vol. 264, no. 24, 25 August 1989, MD US, pages 14408-14414, XP000604828 LIN, S.-H. & GUIDOTTI, G.: "Cloning and expression of a cDNA coding for a rat liver plasma membrane ecto-ATPase"	1,2,4,5
Y	---	3,6-27
X	EUROPEAN JOURNAL OF BIOLOGICAL CHEMISTRY (MICROFILMS), vol. 223, no. 2, 11 July 1994, pages 529-541, XP000603618 HAUCK, W. ET AL.: "Transcriptional control of the human biliary glycoprotein gene, a CEA gene family member down-regulated in colorectal carcinomas" see figure 3	1,3,5,8, 9
X	---	
X	CANCER RESEARCH, vol. 55, no. 6, 15 March 1995, MD US, pages 1215-1220, XP000604808 KLEINERMAN, D.I. ET AL.: "Consistent expression of an epithelial Cell Adhesion Molecule (C-CAM) during human prostate development and loss of expression in prostate cancer: implication as a tumor suppressor" see the whole document	29
Y	---	1-27
Y	WO,A,95 02697 (RHONE-POULENC RORER) 26 January 1995 see claims 1-5,13,14,17,28-30	1-27

	-/-	

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/10696

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>CANCER RESEARCH, vol. 55, no. 13, 1 July 1995, MD US, pages 2831-2836, XP000604839 KLEINERMAN D.I. ET AL.: "Application of a tumor suppressor (C-CAM1)-expressing recombinant adenovirus in androgen-independent human prostate cancer therapy: a preclinical study" see the whole document</p> <p>-----</p>	1-27

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/10696

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 20-26 and partially claims 11-19, as far as they concern an in vivo method, are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/10696

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9502697	26-01-95	FR-A-	2707664	20-01-95
		FR-A-	2718749	20-10-95
		AU-A-	7264694	13-02-95
		CA-A-	2144040	26-01-95
		CN-A-	1113390	13-12-95
		CZ-A-	9500639	15-11-95
		EP-A-	0667912	23-08-95
		FI-A-	951138	13-04-95
		HU-A-	72558	28-05-96
		JP-T-	8501703	27-02-96
		NO-A-	950939	10-03-95
		NZ-A-	269156	26-03-96
		PL-A-	308122	24-07-95
		ZA-A-	9405012	20-02-95